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(71) Applicant: WISCONSIN ALUMNI RESEARCH FOUNDATION [US/US]; 614 North Walnut Street, Madison, WI 53705 (US).

(72) Inventors: PAUZA, Charles, David ; 13404 Barbados Way, Del Mar, CA 92014 (US). DEFTOS, Leonard, John ; 12912 Via Grimaldi, Del Mar, CA 92014 (US). DELUCA, Hector, Floyd ; 1809 Highway BB, Deerfield, WI 53531 (US).

(74) Agent: WOZNY, Thomas, M.; Andrus, Sceales, Starke & Sawall, 735 North Water Street, Suite 1102, Milwaukee, WI 53202 (US).

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(54) Title: NOVEL USE OF VITAMIN D COMPOUNDS TO INHIBIT REPLICATION OF THE AIDS VIRUS

(57) Abstract

A novel use for the Vitamin D compound, 1α, 25-dihydroxycholecalciferol, and its related and derived analogs, to treat human acquired immunodeficiency syndrome, AIDS, inasmuch as these agents inhibit the replication in human cells of HIV, the human immunodeficiency virus that causes the acquired immunodeficiency syndrome, AIDS.

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NOVEL USE OF VITAMIN D COMPOUNDS TO INHIBIT REPLICATION OF THE AIDS VIRUS

Background of the Invention

The present invention relates to vitamin D compounds and more particularly to the use of vitamin D compounds to treat acquired immune deficiency syndrome (AIDS) inasmuch as they inhibit replication of the acquired immune deficiency syndrome (AIDS) virus.

The effects of Vitamin D compounds on the immune system have only recently been recognized. Rigby, Immunology Today, Vol. 9, pages 54-57, 1988 and Manolagas et al Annals of Internal Medicine, Vol. 100, pages 144-146, 1984. This group of compounds is best recognized for their use in disorders of calcium and skeletal metabolism. Manolagas et al, Annals of Internal Medicine, Vol. 100, pages 144-146, 1984 and Manolagas et al, Journal of Clinical Endocrinology and Metabolism, Vol. 63, pages 394-400, 1986. The active Vitamin D metabolite, 1α,25-dihydroxycholecalciferol (1,25DHCC) supports immune function in many in vitro systems. Manolagas et al, Annals of Internal Medicine, Vol. 100, pages 144-146, 1984 and Provvedini et al, Bone, Vol. 7, pages 23-28, 1986. Its actions are pleiotropic, involving most types of immune cells and many of their immunoregulatory cytokines. Rigby, Immunology Today, Vol. 9, pages 54-57, 1988 and Provvedini et al, Bone, Vol. 7, pages 23-28, 1986. One action of 1,25DHCC is to promote the differentiation of monocytes to macrophages and, thus, increase their activity as effector cells of the immune system. Provvedini et al, Bone, Vol. 7, pages 23-28, 1986. Other analogs of 1,25DHCC have similar effects. Zhou et al, Blood, Vol. 74, pages 82-93, 1989. Monocytes and macrophages constitute a reservoir for infection and are important participants in the development of human immunodeficiency virus (HIV) infection. Pauza, Cellular Immunology, Vol. 122, pages 414-424, 1988 and Pauza et al, Journal of Virology, Vol. 62, pages 3558-3564, 1988. Accordingly, a series of Vitamin D compounds should be evaluated for their effects

on this virus in a human monocyte/macrophage cell line, the U937 cell line. This line responds to the pro-differentiation effects of certain Vitamin D metabolites and provides a human model for HIV infection. Pauza et al, Journal of Virology, Vol. 62, pages 3558-3564, 1988.

Summary of the Invention

The effects of Vitamin D compounds on the replication of human immunodeficiency virus (HIV) in human cells are described. The physiologically active metabolites, 1α,25-dihydroxycholecalciferol (1,25DHCC), and two of its analogs, 1α,25-dihydroxy-24,24-difluorocholecalciferol (1,25DHDFCC) and 1α,25-dihydroxy-26,27-hexadeuterocholecalciferol (1,25 DHHDCC), inhibited viral replication in a dose-dependent manner. This action was accompanied by a pro-differentiation effect of the compounds on the phenotype and growth of the cells. The results indicate that these vitamin D compounds as well as others having cellular differentiation activity can be useful in treating the acquired immune deficiency syndrome (AIDS).

Accordingly, compositions containing one or more three vitamin D compounds having cell differentiation activity, preferably selected from the group consisting of 1α-hydroxyvitamin D homolog compounds, 19-nor-vitamin D compounds and secosterol compounds, together with a suitable carrier useful in the treatment of human immunodeficiency virus infection and acquired immune deficiency syndrome (AIDS) are described. The treatment may be topical, oral or parenteral. Methods of employing the compositions are also disclosed. The compounds are present in the composition in an amount from about 0.01 μg/gm to about 100 μg/gm of the composition, and may be administered orally or parenterally in dosages of from about 0.01 μg/day to about 100 μg/day.

In one aspect of the invention, compositions containing one or more side chain unsaturated 1α-

hydroxyvitamin D homolog compounds for the treatment of human immunodeficiency virus infection and acquired immune deficiency syndrome (AIDS) are provided. Methods employing these compositions are also provided.

In another aspect of the invention, compositions containing one or more side chain saturated 1α-hydroxyvitamin D homolog compounds for the treatment of human immunodeficiency virus infection and acquired immune deficiency syndrome (AIDS) are provided. Methods employing these compositions are also provided.

In still another aspect of the invention, compositions containing one or more 19-nor-vitamin D compounds for the treatment of human immunodeficiency virus and acquired immune deficiency syndrome (AIDS) are provided. Methods employing these compositions are also provided.

In yet another aspect of the invention, compositions containing one or more secosterol compounds for the treatment of human immunodeficiency virus and acquired immune deficiency syndrome (AIDS) are provided. Methods employing these compositions are also provided.

The compounds disclosed herein unexpectedly provide highly effective treatments without producing unwanted systemic or local side effects.

Brief Description of the Drawings

Fig. 1 is a graph of reverse transcriptase activity of HIV infected U937 cells versus time for three different concentrations of 1,25-dihydroxyvitamin D_3 (1,25 DHCC);

Fig. 2 is a graph of percent inhibition of HIV replication in U937 cells versus concentrations for six different vitamin D compounds;

Fig. 3 is a graph of percent inhibition of HIV replication in U937 cells and percent cellular differentiation versus concentrations for three different vitamin D compounds;

Fig. 4 illustrates hybridization analysis of viral RNA in vitamin D_3 treated U937 cells; and

Fig. 5 is a graph of percent cellular differentiation versus concentration for 1,25-$(OH)_2D_3$ and three of its homologs.

Fig. 6 illustrates various structures of the vitamin D metabolites and analogs tested.

Fig. 7 illustrates the ability of OH analogs to induce HL-60 cell differentiation as assayed by nitroblue tetrazolium reduction.

Fig. 8 illustrates the induction of phagocytic activity in HL-60 cells by a series of 25-OH-D_3 and 25-OH-D_2 metabolites and analogs.

Fig. 9 illustrates the effect of side chain elongation and/or truncation and 5,6-isomerization on the ability of the 1,25-$(OH)_2D_3$ analog to induce nonspecific acid esterase activity in HL-60 cells.

Fig. 10 illustrates the activity of short chain and primary alcohol analogs of 1,25-$(OH)_2D_3$ in inducing NBT reducing activity in HL-60 cells after a 4-day incubation.

Fig. 11 illustrates the nonspecific acid esterase activity induced by 1,24R,25-$(OH)_3D_3$ and 1,25-$(OH)_2D_2$ in HL-60 cells.

Detailed Description of the Invention

The following analogs of Vitamin D_3 (Myelodysplastic syndromes. Uchino et al, eds, Elsevier, pp. 133-138, 1988) were evaluated for their effect on HIV replication in infected U937 cell cultures. A: 1,25-dihydroxycholecalciferol (1,25DHCC), B: 1,25-dihydroxy-24,24 difluorocholecalciferol (1,25DHFCC), C: 1,25-dihydroxy-26,27-hexadeuterocholecalciferol (1,25 DHHDCC), D: 25-hydroxycholecalciferol (25HCC), E: 24R,25-dihydroxycholecalciferol (24R,25DHCC), and F: 25S,26-dihydroxycholecalciferol (25S,25DHCC).

Cell free supernates were assayed for reverse transcriptase activity as a measure of virus replication.

Figure 1 illustrates that 1,25-dihydroxycholecalciferol (1,25DHCC) inhibits HIV-1 replication in U937 cells. Growing U937 cells were infected with the LAV1 strain of HIV-1 at a multiplicity of infection of 1.0 tissue culture infectious doses per cell as described previously in Pauza, Journal of Virology, Vol. 62, pages 3558-3564, 1988. After 2.5 hours exposure to HIV, the cells were washed to remove extracellular virus and then cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1,25DHCC. Under these conditions of infection, approximately 70% of the cells were immunofluorescence-positive for viral gp120 or p24 on day 3 post-infection in the absence of 1,25DHCC. Samples were removed after various intervals post-infection and the cell-free reverse transcriptase activity was determined as described in Pauza, Journal of Virology, Vol. 622, pages 3558-3564, 1988. After sampling on day 2, the cultures were supplemented with an equal volume of medium containing an appropriate concentration of 1,25DHCC. The filled circles represent data for untreated, control infections. Open circles correspond to 10^{-10}M, filled triangles to 10^{-8}M and open triangles to 10^{-8}M 1,25DHCC respectively. These results represent the average of five independent determinations.

As shown in Fig. 1, 1,25DHCC inhibited viral replication at all doses tested. The effect was dose dependent and curves representing the time course of virus production shifted to the right (Figure 1). At 10^{-9}M drug concentration, the peak of virus production was observed on day 3, compared to the peak on day 2 in untreated cultures and was reduced by approximately 3-fold. Little evidence for HIV production was observed in the 10^{-9}M samples. At 10^{-10}M virus replication became restricted on day 2, similar to control cultures, although the magnitude of this peak was reduced sharply. Accordingly, the dose-response relationship between

1,25DHCC treatment and HIV replication in U937 cells revealed two independent activities of this agent: a quantitative inhibition of virus growth and a qualitative change in the pattern of virus replication. Both of these features attest to the ability of 1,25DHCC to inhibit acute HIV infection of U937 cells.

Based on this pattern of HIV replication in 1,25DHCC treated cultures, day 2 post-infection was used to compare the effects of Vitamin D compounds on HIV replication in U937 cells. Cells were infected in batches for 2.5 hours and then washed and distributed to individual flasks containing the appropriate concentration of drug. Figure 2 illustrates a comparison of the dose-response curves for six analogues of Vitamin D_3, tested for their ability to inhibit HIV replication in U937 cells. In this case, samples were collected at day 2 post-infection. HIV infection and reverse transcriptase assays were as described in Fig. 1.

Figure 2 shows that two 1,25DHCC analogues, namely, 1,25DHDFCC and 1,25DHHDCC, inhibited HIV replication more than 1,25DHCC, itself. Significant inhibition of HIV replication was still observed at concentrations as low as 10^{-10}M of these compounds. The compounds 25HCC and 24R,25DHCC were only slightly effective at reducing HIV production, even at the highest concentrations tested. The 25S,26DHCC analog was marginally inhibitory, although its effective concentration was approximately two orders of magnitude higher than the effective concentration of 1,25 DHCC.

Based on the comparison of these six analogues of Vitamin D, only the first three were believed to be sufficiently active for further investigation. Accordingly, the capacity of these analogues to induce differentiation of the U937 cells was examined and compared to the dose-response curves for inhibition of virus production. Figure 3 illustrates the effects of 1,25-dihydroxycholecalciferol (1,25DHCC),

1,25-dihydroxyhexadeuterocholecalciferol (1,25DHHDCC) and 1,25-dihydroxydifluorocholecalciferol (1,25DHDFCC) on U937 differentiation and HIV replication. Figure 3 illustrates the effect of these metabolites on two measures of cellular differentiation, i.e., the expression of tetrazolium reductase and inhibition of cellular proliferation. The results in Figure 3 represent the average of three independent experiments. The filled circles designate the capacity of these compounds to inhibit HIV replication as assessed by cell-free reverse transcriptase activity on day 2 post-infection. The filled squares show the percentage of cells positive for tetrazolium reductase activity as evaluated by in situ cytologic assay (See Provvedini et al, Bone, Vol. 7 pages 23-28, 1986). Cellular proliferation was also inhibited by these compounds. The extent of inhibition was roughly the same for each of the drugs. The data for 1,25DHCC are shown here and are representative of the effects of the other two analogues. The drug concentrations giving half-maximal responses in these assays were assessed from this graph. The average of the maximum and minimum responses were calculated and the drug concentrations required to induce this effect were determined and tabulated in Table 1.

These results demonstrate that the pro-differentiation effects of the Vitamin D compounds parallel their inhibition of HIV replication. However, the antiviral and pro-differentiation effects could be distinguished by calculating the half-maximal analog concentration necessary for each effect. Similar concentrations were required to affect HIV replication or cellular differentiation. It is thus likely that inhibition of HIV replication was achieved by a mechanism related to the effects on cellular differentiation.

Table 1

Doses Required to Induce Half-Maximal Cellular Differentiation+ or to Inhibit HIV Replication++ in U937 Cells

Compound	Reductase Activity Inhibition	Growth Inhibition	HIV
1,25-DHCC	1.4×10^{-10}M	1.0×10^{-10}M	5.4×10^{-9}M
1,25-DHHDCC	2.6×10^{-10}M	2.3×10^{-10}M	5.0×10^{-9}M
1,25-DHDFCC	4.7×10^{-9}M	7.6×10^{-10}M	1.2×10^{-9}M

+Differentiation was measured as the acquisition of capacity to reduce tetrazolium dye in situ (as described in Provvedini et al, Bone, Vol. 7, pages 23-28, 1986). The half-maximal concentration is determined to be that concentration of drug able to induce 50% of the difference between untreated and cells treated with 10^{-8}M drug. The values for inhibition of cellular differentiation were calculated similarly.

++HIV replication was determined as described with respect to Figure 1. The 50% inhibitory dose was determined graphically as described above.

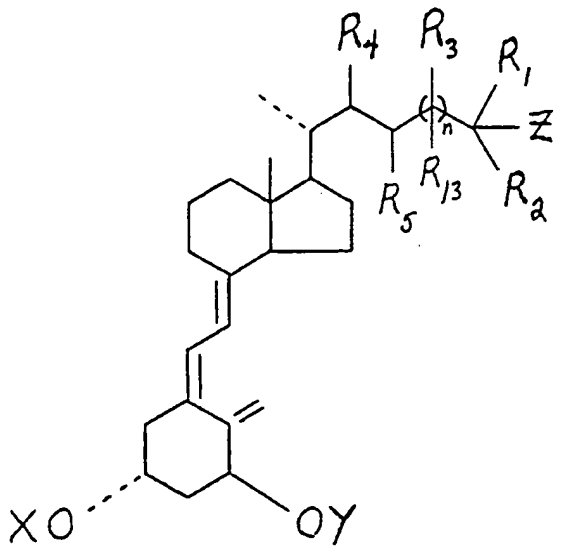
To assess the extent of virus replication in acutely infected U937 cells, analysis of viral RNA accumulation in the cells was performed by Northern hybridization. Figure 4 illustrates hybridization analysis of viral RNA in Vitamin D_3-treated U937 cells. Infections were performed as described with respect to Fig. 1. Time course analyses showed that the peak of viral RNA production occurred on day 2 post-infection; accordingly, this sampling interval was chosen. Total cellular RNA was prepared by guanidinium-isothiocyanate extraction and gradient purification. The purified samples were dissolved in water and the concentration of nucleic acid was determined from the optical density at 260 nm. Twenty micrograms of each RNA was loaded per lane. The 9.2, 4.3 and 2.0 kb HIV RNA species were detected by hybridization with a LTR-specific fragment,

and their positions are indicated on Figur 4. After autoradiographic exposure to reveal the pattern of HIV-1 gene expression, the filters were stripped and rehybridized with a mouse β-actin probe. The intensity of β-actin mRNA serves as a control.

The Vitamin D compounds thus tested produced a decrease in the HIV ribonucleic acid (RNA) of the treated cells, i.e. the intensity of hybridization in the Vitamin D-treated cultures was approximately one-half that observed in the untreated control culture. This value is consistent with the decreased virus production demonstrated in Figure 1.

As a result of the above evaluations, and based upon the demonstrated relationship between cellular differentiation and inhibition of HIV replication, various other vitamin D compounds are expected to have the same or similar therapeutic activity. The vitamin D compounds useful in the compositions of the present invention and for the treatment of acquired immune deficiency syndrome (AIDS) are those which induce cellular differentiation, and preferrably those which induce cellular differentiation with minimal or no effect on either intestinal calcium absorption or bone calcium mobilization. Accordingly, specific preferred examples of vitamin D compounds defined by the above functions are those selected from the group consisting of 1α-hydroxyvitamin D homolog compounds, 19-nor vitamin D compounds and secosterol compounds.

The 1α-hydroxyvitamin D homolog compounds useful in the present invention are characterized structurally as side chain unsaturated and side chain saturated homologs of vitamin D, and preferably of 1,25-$(OH)_2D_3$ in which the side chain is elongated by insertion of one or more methylene units into the chain at the carbon 24 position. They may be represented, therefore, by the following general structure of formula I:

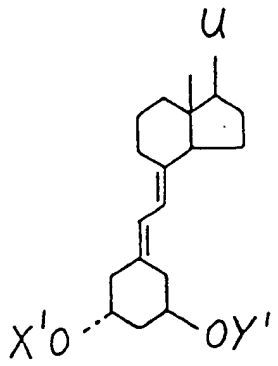


I

where R_4 and R_5 represent hydrogen, deuterium, flourine or when taken together R_4 and R_5 represent a carbon-carbon double bond or a carbon-carbon triple bond, R_{13} represents hydrogen, deuterium, hydroxy, protected hydroxy, fluorine or an alkyl group, Z represents hydrogen, hydroxy or protected-hydroxy, R_3 represents hydrogen, hydroxy, protected hydroxy, fluorine or an alkyl group, X and Y which may be the same or different are hydrogen or a hydroxy-protecting group, R_1 represents the group $-CF_3$, $-CD_3$, or $-(CH_2)_q-H$ and R_2 represents the group $-CF_3$, $-CD_3$, or $-(CH_2)_p-H$, and where n, q and p are integers having independently the values of 1 to 5, and R_1 and R_2 when taken together represent the group $-(CH_2)_m-$ where m is an integer having the value of 2 to 5.

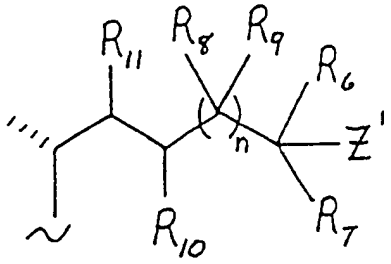
The 19-nor-vitamin D compounds referred to herein are a class of 1α-hydroxylated vitamin D compounds in which the ring A exocyclic methylene group (carbon 19) typical of all vitamin D systems has been removed and replaced by two hydrogen atoms. Structurally these novel

analogs are characterized by the general formula II shown below:



II

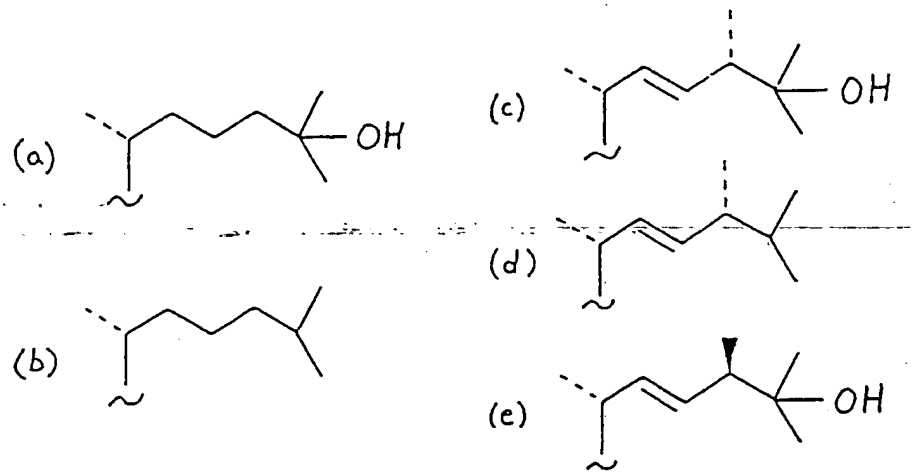
where X^1 and Y^1 are each selected from the group consisting of hydrogen, acyl, alkylsilyl and alkoxyalkyl, and where the group U represents any of the typical side chains known for vitamin D compounds. Thus, U may be an alkyl, hydrogen, hydroxyalkyl or fluoroalkyl group, or U may represent the following side chain:



wherein Z^1 represents hydrogen, hydroxy or O-acyl, R_6 and R_7 are each selected from the group consisting of alkyl, hydroxyalkyl and fluoroalkyl, deuteroalkyl or, when taken together represent the group -- $(CH_2)_m$ -- where m is an integer having a value of from 2 to 5, R_8 is selected from the group consisting of hydrogen, deuterium, hydroxy, fluorine, O-acyl, alkyl, hydroxyalkyl and

fluoroalkyl, R_9 is selected from the group consisting of hydrogen, deuterium, fluorine, alkyl, hydroxyalkyl and fluoroalkyl, or, R_8 and R_9 taken together represent double-bonded oxygen or double-bonded carbon, R_{10} and R_{11} are each selected from the group consisting of hydrogen, deuterium, hydroxy, O-acyl, fluorine and alkyl, or, R_{10} and R_{11} taken together form a carbon-carbon double bond or a carbon-carbon triple bond, and wherein n is an integer having a value of from 1 to 5, and wherein the carbon at any one of positions 20, 22, or 23 in the side chain may be replaced by an O, S, or N atom.

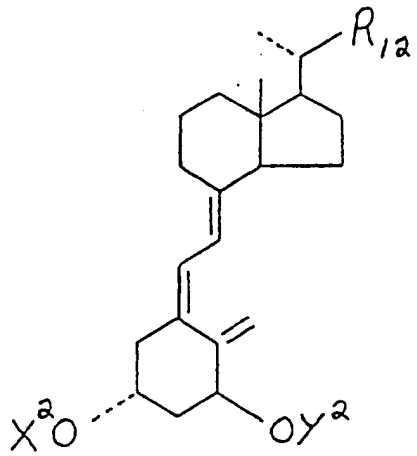
Specific important examples of side chains for the 19-nor compounds are the structures represented by formulas (a), (b), (c), (d) and (e) below, i.e. the side chain as it occurs in 25-hydroxyvitamin D_3 (a); vitamin D_3 (b); 25-hydroxyvitamin D_2 (c); vitamin D_2 (d); and the C-24-epimer of 25-hydroxyvitamin D_2 (e).



Purely structurally, the class of secosterol compounds referred to herein has a similarity with some of the known vitamin D compounds. Unlike the known vitamin D compounds, however, the secosterols used in the present invention do not express the classic vitamin D activities <u>in vivo</u>, i.e. stimulation of intestinal calcium transport, or the mobilization of bone calcium, and hence they cannot be classified as vitamin D derivatives from the functional point of view. In light

of the prior art, it was all the more surprising and unexpected then, to find that these secosterols are remarkably effective in the treatment of AIDS. This finding provides an effective method for the treatment of AIDS, since the above described secosterols can be administered to subjects in doses sufficient to inhibit HIV replication, without producing simultaneously unphysiologically high and deleterious blood calcium levels.

The group of secosterols exhibiting this unique and heretofore unrecognized activity pattern is characterized by the general structure III shown below:



III

where R_{12} is hydrogen, methyl, ethyl or propyl and where each of X^2 and Y^2 represent, independently, hydrogen, an acyl group, or a hydroxy-protecting group.

As used in the description, and in the claims, the term "hydroxy-protecting group" refers to any group commonly used for the protection of hydroxy functions during subsequent reactions, including, for example, acyl or alkylsilyl groups such as trimethylsilyl, triethylsilyl, t-butyldimethylsilyl and analogous alkylated silyl radicals, or alkoxyalkyl groups such as methoxymethyl, ethoxymethyl, methoxyethoxymethyl, tetrahydrofuranyl or tetrahydropyranyl. A "protected-hydroxy" is a hydroxy function derivatized by one of the

above hydroxy-protecting groupings. "Alkyl" represents a straight-chain or branched hydrocarbon radical of 1 to 10 carbons in all its isomeric forms, such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, pentyl, etc., and the terms "hydroxyalkyl" and "fluoroalkyl" refer to such an alkyl radical substituted by one or more hydroxy or fluoro groups respectively. An acyl group is an alkanoyl group of 1 to 6 carbons in all its isomeric forms, or an aroyl group, such as benzoyl, or halo-, nitro- or alkyl-substituted benzoyl groups, or a dicarboxylic acyl group such as oxalyl, malonyl, succinoyl, glutaroyl, or adipoyl. The term "aryl" signifies a phenyl-, cr an alkyl-, nitro- or halo-substituted phenyl group.

It should be noted in this description that the term "24-dihomo" refers to the addition of two methylene groups at the carbon 24 position in the side chain, and the term "trihomo" refers to the addition of three methylene groups at the same position so that both additions have the effect of extending the length of the side chain. Also, the term "26,27-dimethyl" refers to the addition of a methyl group at the carbon 26 and 27 positions so that for example R_1 and R_2 are ethyl groups. Likewise, the term "26,27-diethyl" refers to the addition of an ethyl group at the 26 and 27 positions so that R_1 and R_2 are propyl groups.

Specific and preferred examples of these compounds when the side chain is unsaturated (i.e. R_4 and R_5 represent a double bond) are: 24-dihomo-1,25-dihydroxy-22-dehydrovitamin D_3, i.e. the compound shown above, where X and Y are hydrogen, Z is hydroxy, n equals 3, and R_1 and R_2 are each a methyl group; 26,27-dimethyl-24-dihomo-1,25-dihydroxy-22-dehydrovitamin D_3, i.e. the compound shown above where X and Y are hydrogen, Z is hydroxy, n equals 3, and R_1 and R_2 are each an ethyl group; 24-trihomo-1,25-dihydroxy-22-dehydrovitamin D_3, i.e. the compound having the structure shown above, where X and Y are hydrogen, Z is hydroxy, n equals 4, and R_1

and R_2 are each a methyl group; 26,27-dimethyl-24-trihomo-1,25-dihydroxy-22-dehydrovitamin D_3, i.e. the compound shown above where X and Y are hydrogen, Z is hydroxy, n equals 4, and R_1 and R_2 are each an ethyl group; 26,27-diethyl-24-dihomo-1,25-dihydroxy-22-dehydrovitamin D_3, i.e. the compound shown above where X and Y are hydrogen, Z is hydroxy, n equals 3, and R_1 and R_2 are each a propyl group; 26,27-diethyl-24-trihomo-1,25-dihydroxy-22-dehydrovitamin D_3, i.e. the compound shown above where X and Y are hydrogen, Z is hydroxy, n equals 4, and R_1 and R_2 are each a propyl group, 26,27-dipropyl-24-dihomo-1,25-dihydroxy-22-dehydrovitamin D_3, i.e. the compound shown above where X and Y are hydrogen, Z is hydroxy, n equals 3, and R_1 and R_2 are each a butyl group; and 26,27-dipropyl-24-trihomo-1,25-dihydroxy-22-dehydrovitamin D_3, i.e. the compound shown above where X and Y are hydrogen, Z is hydroxy, n equals 4, and R_1 and R_2 are each a butyl group.

Specific and preferred examples of these compounds when the side chain is saturated (i.e. R_4 and R_5 each represent hydrogen) are: 24-dihomo-1,25-dihydroxy-vitamin D_3, i.e. the compound shown above, where X and Y are hydrogen, Z is hydroxy, n equals 3, and R_1 and R_2 are each a methyl group; 26,27-dimethyl-24-dihomo-1,25-dihydroxy-vitamin D_3, i.e. the compound shown above where X and Y are hydrogen, Z is hydroxy, n equals 3, and R_1 and R_2 are each an ethyl group; 24-trihomo-1,25-dihydroxy-vitamin D_3, i.e. the compound having the structure shown above, where X and Y are hydrogen, Z is hydroxy, n equals 4, and R_1 and R_2 are each a methyl group; 26,27-dimethyl-24-trihomo-1,25-dihydroxy-vitamin D_3, the compound shown above where X and Y are hydrogen, Z is hydroxy, n equals 4, and R_1 and R_2 are each an ethyl group; 26,27-diethyl-24-dihomo-1,25-dihydroxy-vitamin D_3, i.e. the compound shown above where X and Y are hydrogen, Z is hydroxy, n equals 3, and R_1 and R_2 are each a propyl group; 26, 27-diethyl-24-trihomo-1,25-dihydroxy-vitamin

D_3, i.e. the compound shown above where X and Y are hydrogen, Z is hydroxy, n equals 4, and R_1 and R_2 are each a propyl group; 26,27-dipropyl-24-dihomo-1,25-dihydroxy-vitamin D_3, i.e. the compound shown above where X and Y are hydrogen, Z is hydroxy, n equals 3, and R_1 and R_2 are each a butyl group; and 26,27-dipropyl-24-trihomo-1,25-dihydroxy-vitamin D_3, i.e. the compound shown above where X and Y are hydrogen, Z is hydroxy, n equals 4, and R_1 and R_2 are each a butyl group.

Preparation of Homologated Saturated And Unsaturated Side Chain Compounds:

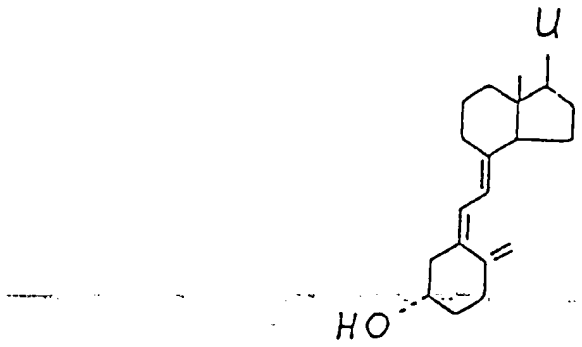
Examples of the compounds of this invention wherein the side chain is saturated can be prepared according to the general process illustrated and described in U. S. Patent No. 4,927,815 issued May 22, 1990 entitled "Compounds Effective In Inducing Cell differentiation And Process For Preparing Same," the description of which is specifically incorporated herein by reference. Examples of the compounds of this invention wherein the side chain is unsaturated can be prepared according to the general process illustrated and described in U. S. Patent No. 4,847,012 issued July 11, 1989 entitled "Vitamin D Related Compounds And Processes For Their Preparation," the description of which is specifically incorporated herein by reference. Examples of the compounds of this invention wherein R_1 and R_2 together represent a cyclopentano group can be prepared according to the general process illustrated and described in U. S. Patent No. 4,851,401 issued July 25, 1989 entitled "Novel Cyclopentano-Vitamin D Analogs," the description of which is specifically incorporated herein by reference.

Another synthetic strategy for the preparation of side-chain-modified analogues of 1α,25-dihydroxyergocalciferol is disclosed in Kutner et al, The Journal of Organic Chemistry, 1988, Vol. 53, pages 3450-3457. In addition, the preparation of 24-homo and 26-

homo vitamin D analogs are disclosed in U. S. Patent No. 4,717,721 issued January 5, 1988 entitled "Sidechain Homo-Vitamin D Compounds With Preferential Anti-Cancer Activity" the description of which is specifically incorporated herein by reference.

Preparation of 19-Nor-Vitamin D Compounds

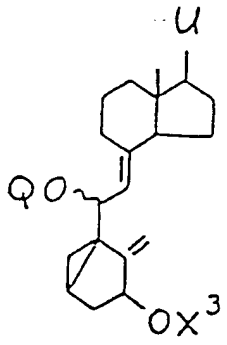
The preparation of 1α-hydroxy-19-nor-vitamin D compounds having the basic structure shown above in formula II can be accomplished by a common general method, using known vitamin D compounds as starting materials. For the synthesis of 1α,25-dihydroxy-19-nor-vitamin D_3, reference is made to Perlman et al, Tetrahedron Letters, 1990, Vol. 31, No. 13, pages 1823-1824. Suitable starting materials are, for example, the vitamin D compounds of the general structure IV:



IV

where U is any of the side chains as defined above. These vitamin D starting materials are known compounds, or compounds that can be prepared by known methods.

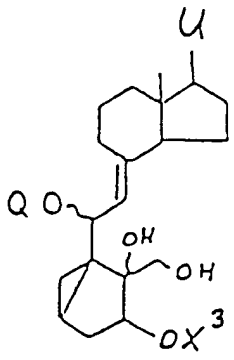
Using the procedure of DeLuca et al U.S. Patent 4,195,027, the starting material is converted to the corresponding 1α-hydroxy-3,5-cyclovitamin D derivative, having the general structure V below, where X^3 represents hydrogen and Q represents an alkyl, preferably methyl:



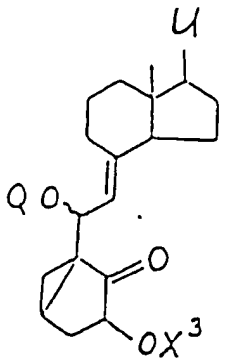
V

So as to preclude undesired reaction of the 1α-hydroxy group in subsequent steps, the hydroxy group is converted to the corresponding acyl derivative, i.e. the compound V shown above, where X^3 represents an acyl group, using standard acylation procedures, such as treatment with an acyl anhydride or acyl halide in pyridine at room temperature or slightly elevated temperature (30-70°C). It should be understood also that whereas the process of this invention is illustrated here with acyl protection of hydroxy functions, alternative standard hydroxy-protecting groups can also be used, such as, for example, alkylsilyl or alkoxyalkyl groups. Such protecting groups are well-known in the art (e.g. trimethylsilyl, triethylsilyl, t.-butyldimethylsilyl, or tetrahydrofuranyl, methoxymethyl), and their use is considered a routine modification of experimental detail within the scope of the process of this invention.

The derivative as obtained above is then reacted with osmium tetroxide, to produce the 10,19-dihydroxy analog, VI (where X^3 is acyl), which is subjected to diol cleavage using sodium metaperiodate or similar vicinal diol cleavage reagents (e.g. lead tetraacetate) to obtain the 10-oxo-intermediate, having the structure VII below (where X^3 is acyl):



VI

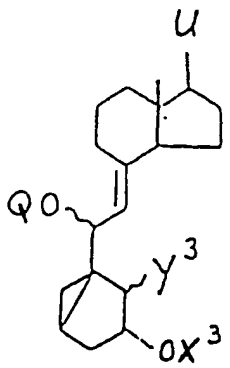


VII

These two consecutive steps can be carried out according to the procedures given by Paaren et al. (J. Org. Chem. 48, 3819 (1983)). If the side chain unit, U carries vicinal diols (e.g. 24,25-dihydroxy- or 25,26-dihydroxy, etc.), these, of course, also need to be protected, e.g. via acylation, silylation, or as the isopropylidene derivative prior to the periodate cleavage reactions.

In most cases, the acylation of the 1α-hydroxy group as mentioned above will simultaneously effect the acylation of side chain hydroxy functions, and these acylation conditions can, of course, be appropriately adjusted (e.g. elevated temperatures, longer reaction times) so as to assure complete protection of side chain vicinal diol groupings.

The next step of the process comprises the reduction of the 10-oxo-group to the corresponding 10-alcohol having the structure VIII shown below (where X^3 is acyl and Y^3 represents hydroxy). When X^3 is acyl, this reduction is carried out conveniently in an organic solvent at from about 0°C to about room temperature, using $NaBH_4$ or equivalent hydride reducing agents, selective for the reduction of carbonyl groups without cleaving ester functions. Obviously, when X^3 is a hydroxy-protecting group that is stable to reducing agents, any of the other hydride reducing agents (e.g. $LiAlH_4$, or analogous reagents) may be employed also.



VIII

The 10-hydroxy intermediate is then treated with an alkyl-or arylsulfonylhalide (e.g. methanesulfonylchloride) in a suitable solvent (e.g. pyridine) to obtain the corresponding 10-0-alkyl-or arylsulfonyl derivative (the compound having the structure shown VIII above, where Y^3 is alkyl-SO_2O-, or aryl-SO_2O-, and this sulfonate intermediate is then directly reduced, with lithium aluminum hydride, or the analogous known lithium aluminum alkyl hydride reagents in an ether solvent, at a temperature ranging from 0°C to the boiling temperature of the solvent, thereby displacing the sulfonate group and obtaining the 10-deoxy derivative, represented by the structure VIII above, where X^3 and Y^3 are both hydrogen. As shown by the above structure, a 1-0-acyl function in the precursor compound VII is also cleaved in this reduction step to produce the free 1α-hydroxy function, and any 0-acyl protecting group in the side chain would, of course, likewise be reduced to the corresponding free alcohol function, as is well understood in the art. If desired, the hydroxy groups at C-1 (or hydroxy groups in the side chain) can be reprotected by acylation or silylation or ether formation to the corresponding acyl, alkylsilyl or alkoxyalkyl derivative, but such protection is not required. Alternative hydroxy-protecting groups, such as alkylsilyl or alkoxyalkyl groups would be retained in this reduction step, but can be removed, as desired, at this or later stages in the process by standard methods known in the art.

structure shown VIII above, where Y^3 is alkyl-SO_2O-, or aryl-SO_2O-, and this sulfonate intermediate is then directly reduced, with lithium aluminum hydride, or the analogous known lithium aluminum alkyl hydride reagents in an ether solvent, at a temperature ranging from 0°C to the boiling temperature of the solvent, thereby displacing the sulfonate group and obtaining the 10-deoxy derivative, represented by the structure VIII above, where X^3 and Y^3 are both hydrogen. As shown by the above structure, a 1-O-acyl function in the precursor compound VII is also cleaved in this reduction step to produce the free 1α-hydroxy function, and any O-acyl protecting group in the side chain would, of course, likewise be reduced to the corresponding free alcohol function, as is well understood in the art. If desired, the hydroxy groups at C-1 (or hydroxy groups in the side chain) can be reprotected by acylation or silylation or ether formation to the corresponding acyl, alkylsilyl or alkoxyalkyl derivative, but such protection is not required. Alternative hydroxy-protecting groups, such as alkylsilyl or alkoxyalkyl groups would be retained in this reduction step, but can be removed, as desired, at this or later stages in the process by standard methods known in the art.

The above 1α-hydroxy-10-deoxy cyclovitamin D intermediate is next solvolyzed in the presence of a low-molecular weight organic acid, using the conditions of DeLuca et al U.S. Patents 4,195,027 and 4,260,549. When the solvolysis is carried out in acetic acid, for example, there is obtained a mixture of 1α-hydroxy-19-nor-vitamin D 3-acetate and 1α-hydroxy-19-nor-vitamin D 1-acetate (compounds IX and X, below), and the analogous 1- and 3-acylates are produced, when alternative acids are used for solvolysis.

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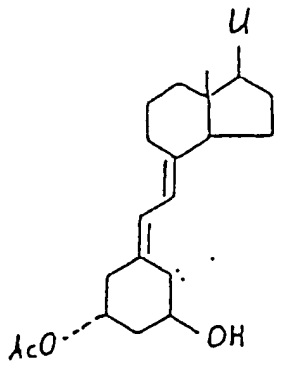
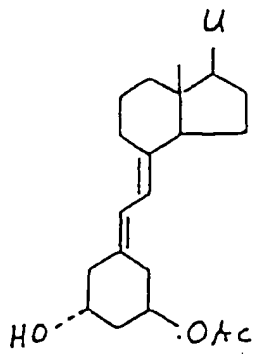


IX



X

Direct basic hydrolysis of this mixture under standard conditions then produces the desired 1α-hydroxy-19-nor-vitamin D compounds of structure II above (where X^1 and Y^1 are both hydrogen). Alternatively, the above mixture of monoacetates may also be separated (e.g. by high pressure liquid chromatography) and the resulting 1-acetate and 3-acetate isomers may be subjected separately to hydroxysis to obtain the same final product from each, namely the 1α-hydroxy-19-nor-vitamin D compounds of structure II. Also the separated monoacetates of structure IX or X or the free 1,3-dihydroxy compound can, of course, be reacylated according to standard procedures with any desired acyl group, so as to produce the product of structure II above, where X^1 and Y^1 represent acyl groups which may be the same or different.

The 19-nor-vitamin D compounds useful in this invention are more specifically described by the following illustrative examples. In these examples specific products identified by Roman numerals and letters, i.e. IIa, IIb, ..., etc. refer to the specific structures and side chain combinations identified in the preceding description.

Example 1

Preparation of 1α,25-dihydroxy-19-nor-vitamin D_3 (IIa)

(a) 1α, 25-Dihydroxy-3,5-cyclovitamin D_3 1-acetate, 6-methyl ether: Using 25-hydroxyvitamin D_3 (IVa) as starting material, the known 1α,25-dihydroxy-3,5-cyclovitamin derivative Va (X^3=H) was prepared according to published procedures (DeLuca et al., U. S. Patent 4,195,027 and Paaren et al., J. Org. Chem. 45, 3252 (1980)). This product was then acylated under standard conditions to obtain the corresponding 1-acetate derivative Va (X^3=Ac).

(b) 10,19-Dihydro-1α,10,19,25-tetrahydroxy-3,5-cyclovitamin $D_3$1-acetate, 6-methyl ether (VIa): Intermediate Va (X^3=Ac) was treated with a slight molar excess of osmium tetroxide in pyridine according to the general procedure described by Paaren et al. (J. Org. Chem. 48, 3819 (1983)) to obtain the 10,19-dihydroxylated derivative VIa. Mass spectrum m/z (relative intensity), 506 (M^+, 1), 488 (2), 474 (40), 425 (45), 396 (15), 285 (5), 229 (30), 133 (45), 59 (80), 43 (100). ^{1}H, NMR ($CDCl_3$) δ 0.52 (3H, s, 18-CH_3, 0.58 (1H, m, 3-H), 0.93 (3H, d, J=6.1 Hz, 21-CH_3, 1.22 (6H, s, 26-CH_3 and 27-CH_3), 2.10 (3H, s, $COCH_3$), 3.25 (3H, s, 6-OCH_3 3.63 (2H, m, 19-CH_2), 4.60 (1H, d, J=9.2 Hz, 6-H), 4.63 (1H, dd, 1β-H), 4.78 (1H, d, J=9.2 Hz, 7-H).

(c) 1α,25-Dihydroxy-10-oxo-3,5-cyclo-19-nor-vitamin D_3 1-acetate, 6-methyl ether (VIIa): The 10,19-dihydroxylated intermediate VIa was treated with a solution of sodium metaperiodate according to the procedure given by Paaren et al. (J. Org. Chem. 48, 3819,

1983) to produce the 10-oxo-cyclovitamin D derivative (VIIa, X^3=Ac). Mass spectrum m/z (relative intensity) 442 (M^+-MeOH) (18), 424 (8), 382 (15), 364 (35), 253 (55), 225 (25), 197 (53), 155 (85), 137 (100). ^{1}H NMR ($CDCl_3$) δ 0.58 (3H, s, 18-CH_3), 0.93 (3H, d, J=6.6 Hz, 21-CH_3), 1.22 (6H, s, 26-CH_3 and 27-CH_3), 2.15 (s, 3-$OCOCH_3$), 3.30 (3H, s, 6-OCH_3), 4.61 (1H, d, J=9.1 Hz, 6-H), 4.71 (1H, d, J=9.6 Hz, 7-H), 5.18 (1H,m, 1β-H).

It has been found also that this diol cleavage reaction does not require elevated temperatures, and it is, indeed, generally preferable to conduct the reaction at approximately room temperature.

(d) <u>1α-Acetoxy-10,25-dihydroxy-3,5-cyclo-19-nor-vitamin D_3 6-methyl ether (VIIIa, X^3=Ac, Y^3=OH)</u>: The 10-oxo derivative VIIa (X^3=Ac) (2.2 mg, 4.6 μmol) was dissolved in 0.5 ml of ethanol and to this solution 50 μl (5.3 μmol) of a $NaBH_4$ solution (prepared from 20 mg of $NaBH_4$, 4.5 ml water and 0.5 ml of 0.01 N NaOH solution) was added and the mixture stirred at 0°C for ca. 1.5 h, and then kept at 0°C for 16 h. To the mixture ether was added and the organic phase washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography on a 15 x 1 cm silica gel column and the alcohol VIIIa (X^3=Ac, Y^3=OH) was eluted with ethyl acetate hexane mixtures to give 1.4 mg (3 μmol) of product. Mass spectrum m/z (relative intensity) 476 (M^+) (1), 444 (85), 426 (18), 384 (30), 366 (48), 351 (21), 255 (35), 237 (48), 199 (100), 139 (51), 59 (58).

(e) <u>1α,25-Dihydroxy-19-nor-vitamin D_3 (IIa, X^1= Y^1=H)</u>: The 10-alcohol (VIIIa, X^3=Ac, Y^3=OH) (1.4 mg) was dissolved in 100 μl anhydrous CH_2Cl_2 and 10 μl (14 μmol) triethylamine solution (prepared from 12 mg (16 μl) triethylamine in 100 μl anhydrous CH_2Cl_2), followed by 7 μl (5.6 μmol) methyl chloride solution (9 mg mesyl chloride, 6.1 μl, in 100 μl anhydrous CH_2Cl_2) added at 0°C. The mixture was stirred at 0°C for 2 h. The

solvents were removed with a stream of argon and the residue (comprising compound VIIIa, X^3=Ac, Y^3=CH_3SO_2O-) dissolved in 0.5 ml of anhydrous tetrahydrofuran; 5 mg of $LiAlH_4$ was added at 0°C and the mixture kept at 0°C for 16 h. Excess $LiAlH_4$ was decomposed with wet ether, the ether phase was washed with water and dried over $MgSO_4$, filtered and evaporated to give the 19-nor product VIIIa (X^3=Y^3=H).

This product was dissolved in 0.5 ml of acetic acid and stirred at 55°C for 20 min. The mixture was cooled, ice water added and extracted with ether. The other phase was washed with cold 10% sodium bicarbonate solution, brine, dried over $MgSO_4$, filtered and evaporated to give the expected mixture of 3-acetoxy-1-α-hydroxy- and 1α-acetoxy-3-hydroxy isomers, which were separated and purified by HPLC (Zorbax Sil column, 6.4 x 25 cm, 2-propanol in hexane) to give about 70 μg each of compounds IXa and Xa. UV (in EtOH) λ_{max} 242.5 (OD 0.72), 251.5 (OD 0.86), 260 (OD 0.57).

Both 19-nor-1,25-dihydroxyvitamin D_3 acetates IXa and Xa were hydrolyzed in the same manner. Each of the monoacetates was dissolved in 0.5 ml of ether and 0.5 ml 0.1 N KOH in methanol was added. The mixture was stirred under argon atmosphere for 2 h. More ether was added and the organic phase washed with brine, dried over anhydrous $MgSO_4$, filtered and evaporated. The residue was dissolved in a 1:1 mixture of 2-propanol and hexane and passed through a Sep Pak column and washed with the same solvent. The solvents were evaporated and the residue purified by HPLC (Zorbax Sil, 6.4 x 25 cm, 10% 2-propanol in hexane). The hydrolysis products of IXa and Xa were identical and gave 66 μg of IIa (X^1=Y^1=H). Mass spectrum (mz relative intensity) 404 (M^+) (100), 386 (41), 371 (20), 275 (53), 245 (51), 180 (43), 135 (72), 133 (72), 95 (82), 59 (18), exact mass calcd. for $C_{26}H_{44}O_3$ 404.3290, found 404.3272. ^1H NMR ($CDCl_3$) δ 0.52 (3H, s, 18-CH_3), 0.92 (3H, d, J=6.9 Hz, 21-CH_3), 1.21 (6H, s, 26-

CH_3 and 27-CH_3), 4.02 (1H, m, 3αH), 4.06 (1H, m, 1β-H), 5.83 (1H, d, J=11.6 Hz, 7-H), 6.29 (1H, d, J=10.7Hz, 6-H). UV (in EtOH), λ_{max} 243 (OD 0.725), 251.5 (OD 0.823), 261 (OD 0.598).

Example 2

Preparation of 1α-hydroxy-19-nor-vitamin D_3(IIb):

(a) With vitamin D_3 (IVb) as starting material, and utilizing the conditions of Example 1a, there is obtained known 1α-hydroxy-3,5-cyclovitamin D_3 1-acetate, 6-methyl ether, compound Vb (X^3=Ac).

(b) By subjecting intermediate Vb (X^3=Ac), as obtained in Example 2a above to the conditions of Example 1b, there is obtained 10,19-dihydro-1α,10-19-trihydroxy-3,5-cyclovitamin D_3 1-acetate, 6-methyl ether VIb (X^3=Ac).

(c) By treatment of intermediate VIb (X^3=Ac) with sodium metaperiodate according to Example 1c above, there is obtained 1α-hydroxy-10-oxo-3,5-cyclo-19-nor-vitamin D_3 1-acetate, 6-methyl ether VIIb (X^3=Ac).

(d) Upon reduction of the 10-oxo-intermediate VIIb (X^3=Ac) under the conditions of Example 1d above, there is obtained 1α-acetoxy-10-hydroxy-3,5-cyclo-19-nor-vitamin D_3 6-methyl ether VIIIb (X^3=Ac, Y^3=OH).

(e) Upon processing intermediate VIIIb (X^3=Ac, Y^3=OH) through the procedure given in Example 1e above, there is obtained 1α-hydroxy-19-nor-vitamin D_3 (IIb, X^1=Y^1=H).

Example 3

Preparation of 1α,25-dihydroxy-19-nor-vitamin D_2:

(a) Utilizing 25-hydroxyvitamin D_2 (IVc) as starting material and experimental conditions analogous to those of Example 1a, there is obtained 1α,25-dihydroxy-3,5-cyclovitamin D_2 1-acetate, 6-methyl ether, compound Vc (X^3=Ac).

(b) Subjecting intermediate Vc (X^3=Ac), as obtained in Example 3a above, to the reaction conditions of Example 1b, provides 10,19-dihydro-1α,10,19,25-tetrahydroxy-3,5-cyclovitamin D_2 1-acetate, 6-methyl ether, VIc (X^3=Ac).

(c) By treatment of intermediate VIc (X^3=Ac) with sodium metaperiodate according to general procedures of Example 1c above, there is obtained 1α,25-dihydroxy-10-oxo-3,5-cyclo-19-nor-vitamin D_2 1 acetate, 6-methyl ether VIIc (X^3=Ac).

(d) Upon reduction of the 10-oxo-intermediate VIIc (X^3=Ac) under conditions analogous to those of Example 1d above, there is obtained 1α-acetoxy-10,25-dihydroxy-3,5-cyclo-19-nor-vitamin D_2 6-methyl ether VIIIc (X^3=Ac, Y^3=OH).

(e) Upon processing intermediate VIIIc (X^3=Ac, Y^3=OH) through the procedural steps given in Example 1e above, there is obtained 1α,25-dihydroxy-19-nor-vitamin D_2 (IIc, X^1=Y^1=H).

Example 4

Preparation of 1α-hydroxy-19-nor-vitamin D_2:

(a) With vitamin D_2 (IVd) as starting material, and utilizing the conditions of Example 1a, there is obtained known 1α-hydroxy-3,5-cyclovitamin D_2 1-acetate, 6-methyl ether, compound Vd (X^3=Ac).

(b) By subjecting intermediate Vd (X^3=Ac), as obtained in Example 4a above to the conditions of Example 1b, there is obtained 10,19-dihydro-1α,10,19-trihydroxy-3,5-cyclovitamin D_2 1-acetate, 6-methyl ether, VId (X^3=Ac).

(c) By treatment of intermediate VId (X^3=Ac) with sodium metaperiodate according to Example 1c above, there is obtained 1α-hydroxy-10-oxo-3,5-cyclo-19-nor-vitamin D_2 1-acetate, 6-methyl ether, VIId (X^3=Ac).

(d) Upon reduction of the 10-oxo-intermediate VIId (X^3=Ac) under the conditions of Example 1d above, there is obtained 1α-acetoxy-10-hydroxy-3,5-cyclo-19-nor-vitamin D_2 6-methyl ether, VIIId (X^3=Ac, Y^3=OH).

(e) Upon processing intermediate VIIId (X^3=Ac, Y^3=OH) through the procedure given in Example 1e above, there is obtained 1α-hydroxy-19-nor-vitamin D_2 (IId, X^1=Y^1=H).

Preparation of Secosterol Compounds:

The secosterol of structure III where R_{12} is hydrogen can be prepared according to the method of Lam et al as published in Steroids 26, 422 (1975), the description of which is specifically incorporated herein by reference. The secosterols of structure III, where R_{12} is methyl, ethyl or propyl, can be prepared according to the general process illustrated and described in U. S. Patent No. 4,800,198 issued January 24, 1989 entitled "Method of Inducing the Differentiation of Malignant Cells With Secosterol", the description of which is specifically incorporated herein by reference.

Compositions for use in the above-mentioned treatment of AIDS comprise an effective amount of one or more vitamin D compounds as defined by the above formulae, and a suitable carrier. The preferred compounds are one or more side chain unsaturated or side chain saturated 1α-hydroxyvitamin D homolog compound, one or more 19-nor-vitamin D compound, or one or more secosterol compound as the active ingredient. An effective amount of such compounds for use in accordance with this invention is from about 0.001 μg to about 10.0 μg per gm of composition, and may be administered topically, orally or parenterally in dosages of from about 0.1 μg/day to about 100 μg/day. A concentration of 0.01 μg per gm of the composition is preferred.

The compounds may be formulated as creams, lotions, ointments, topical patches, pills, capsules or tablets, or in liquid form as solutions, emulsions, dispersions or suspensions in pharmaceutically innocuous and acceptable solvent or oils, and such preparations may contain in addition other pharmaceutically innocuous or beneficial components, such as antioxidants, emulsifiers, coloring agents, binders or coating materials.

The compounds may be administered topically, as oral doses, or parenterally by injection or infusion of suitable sterile solutions. The compounds are

advantageously administered in amounts sufficient to effect the differentiation of Promyelocytes to normal macrophages. Dosages as described above are suitable, it being understood that the amounts given are to be adjusted in accordance with the severity of the disease, and the condition and response of the subject as is well understood in the art.

Biological Activity of 1α-Hydroxyvitamin D Homolog Compounds:

Four different 24-homologated compounds having structures falling within formula I were tested for both differentiation activity and calcemic activity, using established assays known in the art. Differentiation activity was assessed by nonspecific acid esterase (NSE) activity, nitroblue tetrazolium (NBT) reducing activity and phagocytic capacity in HL60 cells. Calcium mobilizing activity was assessed by intestinal calcium transport data and serum calcium data.

Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; Δ^{22}-24,24-dihomo-1,25-$(OH)_2D_3$, (22E)-22-dehydro-24,24-dihomo-1,25-dihydroxyvitamin D_3; Δ^{22}-24,24,24-trihomo-1,25-$(OH)_2D_3$, (22E)-22-dehydro-24,24,24-trihomo-1,25-dihydroxyvitamin D_3; 24-homo-1,25-$(OH)_2D_3$, 24-homo-1,25-dihydroxyvitamin D_3.

Test Results. Data is presented in Table 2 as the percent of differentiated cells resulting from treatment with various concentrations of 1,25-$(OH)_2D_3$ (used as comparison standard) or one of the four homologated vitamin D test compounds. The data in Table 2 is also shown in Figure 5. Calcemic activity of the compounds is presented in Table 3 and expressed in terms of intestinal calcium transport data and serum calcium data.

Table 2: HL-60 Differentiating Activity of 24-Homologues of 1,25-$(OH)_2D_3$[a]

compound	concn (M)	NSE (%)	NBT (%)	phagocytosis (%)
1,25-$(OH)_2D_3$	1×10^{-7}	87 ± 2	85 ± 3	88 ± 3
	1×10^{-8}	61 ± 3	60 ± 2	62 ± 4
	1×10^{-9}	34 ± 2	38 ± 3	34 ± 3
24-homo-1,25-$(OH)_2D_3$	5×10^{-8}	89 ± 2	89 ± 3	90 ± 3
	1×10^{-8}	80 ± 2	82 ± 4	78 ± 2
	5×10^{-9}	68 ± 4	69 ± 3	70 ± 3
	1×10^{-9}	56 ± 3	53 ± 5	49 ± 3
	5×10^{-10}	43 ± 2	40 ± 4	39 ± 2
24,24-dihomo-1,25-$(OH)_2D_3$	5×10^{-8}	90 ± 3	89 ± 5	88 ± 3
	1×10^{-8}	83 ± 3	82 ± 3	80 ± 3
	5×10^{-9}	67 ± 2	65 ± 5	66 ± 2
	1×10^{-9}	49 ± 2	51 ± 4	51 ± 4
Δ^{22}-24,24-dihomo-1,25-$(OH)_2D_3$	5×10^{-8}	92 ± 2	93 ± 3	90 ± 3
	1×10^{-8}	78 ± 3	81 ± 2	80 ± 4
	5×10^{-9}	67 ± 2	68 ± 3	65 ± 3
	1×10^{-9}	49 ± 2	50 ± 4	51 ± 3
	5×10^{-10}	36 ± 4	38 ± 4	37 ± 4
Δ^{22}-24,24,24-trihomo-1,25-$(OH)_2D_3$	1×10^{-7}	79 ± 2	76 ± 2	77 ± 2
	5×10^{-8}	68 ± 2	68 ± 4	54 ± 3
	1×10^{-8}	41 ± 4	36 ± 1	40 ± 2
	5×10^{-9}	25 ± 3	25 ± 2	23 ± 2
	1×10^{-9}	16 ± 2	13 ± 2	16 ± 4

[a] Results are expressed as percent of total cells counted that have differentiated.

Table 3: Calcium Mobilizing Activity of 24-Homologated 1,25-Dihydroxyvitamin D Compounds[a]

expt	compound	dose (pmol/ day)	Ca transport (SEM)	serum Ca (mg/100 mL)
I	–control	0	2.6 ± 0.4^{a}	3.9 ± 0.3^{a}
	$+1,25\text{-}(OH)_2D_3$	6.5	$4.4 \pm 0.6^{b^1}$	
		32.5	$4.8 \pm 0.2^{b^2}$	$4.3 \pm 0.3^{b^2}$
		65	$7.3 \pm 1.9^{b^3}$	$5.1 \pm 0.9^{b^3}$
	24-homo-1,25-$(OH)_2D_3$	6.5	$3.2 \pm 0.4^{c^1}$	
		32.5	$4.6 \pm 0.8^{c^2}$	
		65	$6.4 \pm 1.3^{c^3}$	$4.0 \pm 0.31^{c^3}$
II	control	0	4.8 ± 0.26^{a}	4.1 ± 0.11^{a}
	$1,25\text{-}(OH)_2D_3$	32.5	$11.2 \pm 0.58^{b^1}$	$4.9 \pm 0.2^{b^1}$
		65	$13.4 \pm 1.1^{b^2}$	$4.9 \pm 0.2^{b^2}$
	24,24-dihomo-1,25-$(OH)_2D_3$	285	9.4 ± 0.77^{c}	
		570		$4.2 \pm 0.2^{c^2}$
		1140		$3.6 \pm 0.19^{c^3}$
		2280		$3.8 \pm 0.2^{c^4}$
		0		$3.6 \pm 0.02^{a^1}$
	Δ^{22}-24,24-dihomo-1,25-$(OH)_2D_3$	285	6.8 ± 0.5^{d}	$4.1 \pm 0.1^{d^2}$
		570		$4.1 \pm 0.1^{d^3}$
		1140		$3.8 \pm 0.2^{c^4}$
		2280		$3.6 \pm 0.1^{d^5}$
III	control		5.2 ± 0.23^{a}	4.0 ± 0.1^{a}
	$1.25\text{-}(OH)_2D_3$	600	12.0 ± 1.5^{b}	5.5 ± 0.1^{b}
	Δ^{22}-24,24,24-trihomo-1,25-$(OH)_2D_3$	55		$4.1 \pm 0.3^{c^1}$
		275	$7.4 \pm 0.3^{c^2}$	$3.8 \pm 0.1^{c^2}$
		550	$6.0 \pm 0.4^{c^3}$	$4.0 \pm 0.2^{c^3}$
		1096	$5.9 \pm 0.3^{c^4}$	$4.0 \pm 0.2^{c^4}$

[a] Vitamin D deficient rats were fed a low-calcium diet and given the indicated daily dose of compound in propylene glycol intraperitoneally or by Alzet minipump (experiment I) for 7 days. Controls received the vehicle. At 7 days the rats were killed for the determinations. There were at least six rats per group. Statistical analysis was done by Student's *t* test. Experiment I, Ca transport: b^1, b^2, b^3 from a, $p < 0.025$; c^1, c^2 from a, NS; c^3 from a, $p = 0.025$; c^1c^2, c^3 from b^1, b^2, b^3, NS; b^3 from b^1, b^2, NS. Experiment I, serum Ca: b^3 from a, $p < 0.01$; b^2 from a, NS. Experiment II, Ca transport: b^1, b^2 from a, $p < 0.001$; c from a, $p < 0.001$; d from a, $p < 0.001$; c from b^1, $p < 0.05$; c from b^2, $p = 0.01$; d from b^1, b^2, $p < 0.001$; d from c, $p = 0.01$; b^1 from b^2, NS. Experiment II, serum Ca: b^1, b^2 from a, $p < 0.005$; c^{2-4}, d^{1-5} from a, NS; b^1, b^2 from c^{2-4}, $p < 0.001$; b^1, b^2 from d^{1-5}, $p < 0.001$; d^{1-5} from a^1 and a, NS. Experiment III, Ca transport: b from a, $p = 0.001$; c^2 from a, $p < 0.05$; c^3 and c^4 from a, NS; c^{2-4} from b, $p < 0.01$. Experiment III, serum Ca: b from a, $p < 0.001$; c^{1-4} from a, NS; b from c^{1-4}, $p < 0.001$.

As shown in Figure 5, 24,24-dihomo-1,25-$(OH)_2D_3$ and 24-homo-1,25-$(OH)_2D_3$ are approximately 10 times more active than the native hormone in causing differentiation of HL-60 cells. Thus, the addition of more than one carbon at the carbon 24 position does not increase differentiative activity further. The addition of an additional carbon at the carbon 24 position as in Δ^{22}-24,24,24-trihomo-1,25-$(OH)_2D_3$ results in differentiative activity half that of the native hormone. Table 2 illustrates that other measurements of differentiation activity gave the same result.

The results presented in Table 2 clearly indicate that the four 24-homologated compounds tested are potent in inducing the differentiation of leukemic cells to normal monocyte cells. For example, at a concentration of 1×10^{-8} molar, 1,25-$(OH)_2D_3$ produces 60-62% differentiated cells, whereas 24,24-dihomo-1,25-$(OH)_2D_3$ at the same concentration gives 80-83% differentiation. Considering that a concentration of 1×10^{-7} molar of 1,25-$(OH)_2D_3$ is required to achieve about the same degree of differentiation as produced by a concentration of 1×10^{-8} molar of the dihomo analog, one can conclude that this analog is in the order of 10 times more potent than 1,25-$(OH)_2D_3$ as a differentiation agent.

In sharp contrast, the four 24-homologated compounds show very low calcemic activity compared to 1,25-$(OH)_2D_3$. This conclusion is supported by the results of Tables 2 and 3. The intestinal calcium transport assay, represented by Table 3, for example, shows the known active metabolite, 1,25-$(OH)_2D_3$ to elicit, as expected, very pronounced responses (compared to control) when administered. There is no doubt that 1,25-$(OH)_2D_3$ is the superior compound in terms of mobilizing calcium from the skeleton. 24-Homo-1,25-$(OH)_2D_3$ showed no calcium mobilizing activity from the skeleton when provided at 65 pmol/day, whereas 1,25-$(OH)_2D_3$ elicited calcium mobilizing activity at 65

pmol/day. When provided at as much as 2280 pmol/day, neither of the dihomo compounds elicited a bone calcium mobilization response, whereas significant bone mobilizing response was found with 1,25-$(OH)_2D_3$ provided at 32 pmol/day. These results suggest that the dihomo compounds are approximately 1000 times less active in mobilizing skeletal calcium than is 1,25-$(OH)_2D_3$. Not surprisingly, therefore, no bone calcium mobilization was found with Δ^{22}-24,24,24-trihomo-1,25-$(OH)_2D_3$.

In the case of intestinal calcium transport, 6.5-32.5 pmol/day of 1,25-$(OH)_2D_3$ appears to saturate this system. The 24-homo-1,25-$(OH)_2D_3$ compound is less active than 1,25-$(OH)_2D_3$ in this test. However, the dihomo compounds do not saturate even when provided at 285 pmol and thus are at least 10 times less active than 1,25-$(OH)_2D_3$. The trihomo compound shows little or no activity at even 1096 pmol/day. Although exact estimates of activity in this system are not possible from the data available, it is clear that the dihomo and trihomo compounds are at least 10 times less active in intestinal calcium transport than is 1,25-$(OH)_2D_3$.

In summary, it is evident from Figure 5 and the data in Tables 2 and 3 that Δ^{22}-24,24,24-trihomo-1,25-$(OH)_2D_3$ retains almost full activity (i.e. half that of 1,25-$(OH)_2D_3$) in causing differentiation of HL-60 cells into monocytes, whereas it has lost most of its calcium mobilizing activity. Because some intestinal calcium transport activity is noted at high doses of the dihomo compounds, these compounds should increase serum calcium slightly when calcium is present in the intestine. The 24,24-dihomo-1,25-$(OH)_2D_3$ compounds, whether saturated in the 22-position or unsaturated, have 10-fold higher HL-60 differentiative activity than 1,25-$(OH)_2D_3$ but have markedly diminished calcium mobilizing activity. The 24-homo-1,25-$(OH)_2D_3$ shows a 10-fold increase in the HL-60 activity and a 5-10 fold decrease in calcium mobilizing activity. If the differentiative

activity is of thereapeutic importance in the treatment of AIDS as the data presented herein indicates, then the 24-homologated 1,25-$(OH)_2D_3$ compounds may be very effective.

Biological Activity of 1α-Hydroxy-19-Nor-Vitamin D Compounds

The 19-nor compounds of this invention also exhibit a pattern of biological activity similar to the above homologated compounds, namely, high potency in promoting the differentiation of malignant cells and little or no activity in calcifying bone tissue. This is illustrated by the biological assay results obtained for 1α,25-dihydroxy-19-nor-vitamin D_3 which are summarized in Tables 4 and 5, respectively. Table 4 shows a comparison of the activity of the known active metabolite 1α,25-dihydroxyvitamin D_3 and the 19-nor analog 1α,25-dihydroxy-19-nor-vitamin D_3 in inducing the differentiation of human leukemia cells (HL-60 cells) in culture to normal cells (monocytes). Differentiation activity was assessed by three standard differentiation assays, abbreviated in Table 4 as NBT (nitroblue tetrazolium reduction), NSE (non-specific esterase activity), and PHAGO (phagocytosis activity). The assays were conducted according to known procedures, as given, for example, by DeLuca et al. (U.S. Patent 4,717,721 and Ostrem et al., J. Biol. Chem. 262, 14164, 1987). For each assay, the differentiation activity of the test compounds is expressed in terms of the percent of HL-60 cells having differentiated to normal cells in response to a given concentration of test compound.

The results summarized in Table 4 clearly show that the analog, 1α,25-dihydroxy-19-nor-vitamin D_3 is as potent as 1α,25-dihydroxyvitamin D_3 in promoting the differentiation of leukemia cells. Thus in all three assays close to 90% of the cells are induced to differentiate by 1α,25-dihydroxy-vitamin D_3 at a concentration of 1×10^{-7} molar, and the same degree of

differentiation (i.e. 90, 84 and 90%) is achieved by the 19-nor analog.

Table 4

Differentiation of HL-60 Cells

1α,25-dihydroxyvitamin D_3 (moles/liter)	% Differentiated Cells (mean ± SEM)		
	NBT	NSE	PHAGO
1×10^{-7}	86 ± 2	89 ± 1	87 ± 3
1×10^{-8}	60 ± 2	60 ± 3	64 ± 2
1×10^{-9}	33 ± 2	31 ± 2	34 ± 1
1α,25-Dihydroxy-19-nor-vitamin D_3 (moles/liter)			
2×10^{-7}	94 ± 2	95 ± 3	94 ± 2
1×10^{-7}	90 ± 4	84 ± 4	90 ± 4
5×10^{-8}	72 ± 3	73 ± 3	74 ± 3
1×10^{-8}	61 ± 3	60 ± 3	56 ± 1
1×10^{-9}	32 ± 1	31 ± 1	33 ± 1

In contrast to the preceding results, the 19-nor analog exhibits no activity in an assay measuring the calcification of bone, a typical response elicited by vitamin D compounds. Relevant data, representing the results of an assay comparing the bone calcification activity in rats of 1α,25-dihydroxyvitamin D_3 and 1α,25-dihydroxy-19-nor vitamin D_3 are summarized in Table 5. This assay was conducted according to the procedure described by Tanaka et al., Endocrinology 92, 417 (1973).

The results presented in Table 5 show the expected bone calcification activity of 1α,25-dihydroxyvitamin D_3 as reflected by the increase in percent bone ash, and in total ash at all dose levels. In contrast, the 19-nor analog exhibits no activity at all three dose levels, when compared to the vitamin D-deficient (-D) control group.

Table 5

Calcification Activity

Compound	Amount Administered* (pmoles/day/7 days)	% Ash (mean ± SEM)	Total Ash (mg) (mean ± SEM)
-D (control)	0	19 ± 0.8	23 ± 1.2
1α,25-dihydroxy-vitamin D_3	32.5	23 ± 0.5	34 ± 1.6
	65.0	26 ± 0.7	36 ± 1.1
	325.0	28 ± 0.9	40 ± 1.9
1α,25-dihydroxy-19-nor-vitamin D_3	32.5	22 ± 0.9	28 ± 1.6
	65.0	19 ± 1.5	28 ± 3.4
	325.0	19 ± 1.2	30 ± 2.4

* Each assay group comprised 6 rats, receiving the indicated amount of test compound by intraperitoneal injection daily for a period of seven days.

Thus the 19-nor analog shows a selective activity profile combining high potency in inducing the differentiation of malignant cells with very low or no bone calcification activity. The compounds of this novel structural class, therefore, can be useful as therapeutic agents for the treatment of AIDS.

Biological Properties of Secosterol Compounds

Biological activity of compounds of structure III in the differentiation of human leukemia cells.

Two different secosterol compounds having structures falling within formula III were tested for both differentiation activity and calcemic activity using established assays known in the art. The differentiation results are reported in Table 6.

Table 6

Percent differentiation of HL-60 cells induced by seco-sterols or by known 1α-hydroxyvitamin D compounds administered at various concentrations as measured by NBT-reduction, rosette formation and esterase activity assays

Compound Administered	Concentration (M)	NBT Reduction (%)	Rosette Formation (%)	Esterase Activity (%)
EtOH	Control	4.5	9	3.5
Secosterol III (R_{12}=CH_3, Y^2=X^2=H)	1×10^{-7}	9	9	2
	1×10^{-6}	14	23	11
	5×10^{-6}	59	68	69
Secosterol III (R_{12}=CH_2CH_3, Y^2=X^2=H)	1×10^{-7}	15	23	30
	1×10^{-6}	28	30	77
	1×10^{-5}	69	70	91
1α-OH-D_3	1×10^{-7}	10	44	12
	1×10^{-6}	39	61	90
	1×10^{-5}	85	79	100
1α,25-$(OH)_2D_3$	1×10^{-8}	39	44	65
	1×10^{-7}	83	76	90

The above results illustrate the efficacy of the seco-sterols of general structure III as agents for the differentiation of human leukemia cells to macrophages (monocytes). The compounds show highly significant activity in all three of the differentiation assays used; 50% differentiation is achieved at concentrations of about 10^{-6} M. For comparative purposes, the table above also includes the cell differentiation activity exhibited by 1α-hydroxyvitamin D_3 (1α-OH-D_3) and 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), two known vitamin D derivatives with potent antileukemic action. The tabulated data show that the level of activity of the seco sterols is lower than that shown by 1,25-$(OH)_2D_3$ (the most potent vitamin D-derived agent for differentiation of leukemia cells), but is approximately equivalent to that shown by 1α-hydroxyvitamin D_3, a compound known to be effective in the treatment of human leukemoid diseases (Suda et al., U.S. Patent 4,391,802).

Assay of secosterols of structure III for bone calcium mobilization and calcium transport.

Male weanling rats, purchased from the Holtzman Co., Madison, WI, were fed the low calcium, vitamin D-deficient diet described by Suda et al. [J. Nutr. 100, 1049 (1970)] ad libitum for 3 weeks. The rats were then divided into 4 groups of 6 animals each. The first group (control group) received 0.05 ml of 95% EtOH by intrajugular injection. The second and third groups were dosed by the same route with 625 picomoles and 6250 picomoles, respectively, of secosterol III (R_{12}=CH_3, Y^2=X^2=H) dissolved in 0.05 ml of EtOH, and the fourth group received an intrajugular injection of 625 picomole of 1α,25-dihydroxyvitamin D_3 (in 0.05 ml of EtOH). Seven hours after dosing, the rats were killed by decapitation and their blood was collected and centrifuged to obtain serum. Serum calcium concentration was determined with an atomic absorption spectrometer according to the conventional protocol. Results are listed in Table 7 below.

The small intestines of these same rats were removed, rinsed and everted for measurement of calcium transport activity according to the technique of Martin and DeLuca [Am. J. Physiol. 216, 1351 (1969)]. The measured intestinal calcium transport activity data, expressed as the ratio of serosal/mucosal calcium concentration, are also listed in Table 7.

Table 7

Compound Administered	Amount (pmole)	Serum Calcium Concentration (mg/100 ml) mean ± S.D.	Intestinal Ca-transport [Ca-serosal]/ [Ca-mucosal] mean ± S.D.
EtOH (control)	--	2.6 ± 0.1	3.6 ± 0.1
Secosterol III (R_{12}=CH_3, y^2=X^2=H)	625	2.9 ± 0.1	3.4 ± 0.1
Secosterol III (R_{12}=CH_3, y^2=X^2=H)	6250	3.0 ± 0.1	3.4 ± 0.1
1,25-$(OH)_2D_3$	625	3.8 ± 0.2	6.7 ± 0.8

The above results show that secosterol III (R_{12}=CH_3, y^2=X^2=H) expresses no significant calcemic activity even at high doses. The compound does not elevate serum calcium levels and thus is devoid of significant bone calcium mobilization activity. Further, the compound does not stimulate calcium transport in the intestine at a dose level of 6250 picomole per animal. Under the same conditions, the known active vitamin D metabolite, 1,25-$(OH)_2D_3$, is, as expected, fully active at 10 times lower dose levels.

It can be concluded, therefore, that these seco steroids of general structure III (where R_{12} is hydrogen, methyl, ethyl, propyl) do not carry out the classical vitamin D functions in vivo, since they elicit no significant in vivo biological response with respect to bone mineral mobilization, and intestinal calcium transport activation.

The above data establish that the seco-sterols of this invention possess an unusual and unexpected spectrum of activities. They exhibit highly significant cell differentiation activity, like some of the known vitamin D-related compounds, but do not express the calcemic activity typical of vitamin D-derivatives. Thus, in being devoid of the undesired calcemic action of the known antileukemic vitamin D-compounds, the seco-steroids of this invention provide a novel and preferred method for the treatment of viral diseases such as AIDS.

Bone calcium mobilization activity of 1α,25-$(OH)_2$-26-homo-D_3 compounds

Male weanling rats were purchased from Holtzman Co., Madison, Wis. and fed ad libitum a low calcium, vitamin D deficient diet as described by Suda et al (J. Nutrition 100: 1049, 1970) and water for 3 weeks. The rats were then divided into 4 groups of 6 each and were intrajugularly given respectively 650 pmole of either 1α,25-$(OH)_2$-26-homo-D_3,

1α,25-$(OH)_2$-(22E)Δ^{22}-26-homo-D_3 or 1α,25-$(OH)_2D_3$ dissolved in 0.05 ml of 95% ethanol 7 hrs. prior to sacrifice. The rats in the control group were given 0.05 ml of 95% ethanol 7 hrs. prior to sacrifice. The rats in the control group were given 0.05 ml of ethanol vehicle in the same manner. They were killed by decapitation, the blood was collected and centrifuged to obtain serum. Serum calcium concentration was determined with an atomic absorption spectrophotometer (Perkin-Elmer Model 214) in presence of 0.1% lanthanum chloride. Results are shown in the table below:

Table 8

Compound Administered	Serum Calcium Concentration (mg/100 ml)	
ethanol	3.4 ± 0.3*	a)
1α,25-$(OH)_2$-26-homo-D_3	4.6 ± 0.2	b)
1α,25-$(OH)_2$-(22E)Δ^{22}-26-homo-D_3	4.6 ± 0.3	b)
1α,25-$(OH)_2D_3$	4.5 ± 0.2	b)

*standard deviation from the mean

b) is significantly different from a) P 0.001

It can be concluded from the foregoing data that in the vitamin D responsive systems of vitamin D-deficient animals the compounds of this invention exhibited the same activity as 1α,25-hydroxyvitamin D_3, the circulating hormonal form of the vitamin.

It has recently been discovered that 1α,25-dihydroxy-vitamin D_3 (1α,25-$(OH)_2D_3$) and its structural analog 1α-hydroxyvitamin D_3 (1α-OH-D_3), in addition to their well-established calcemic action referred to above, also express potent anti-cancer activity. Specifically, it was shown that the above-named compounds were effective in causing differentiation of malignant human cells, such as leukemia cells in culture, to non-malignant macrophages, and the anti-cancer activity on cells in vitro could be correlated with beneficial effects in vivo by showing that the administration of these compounds extended the life span of leukemic mice (compared to controls) and markedly improved the condition of human leukemia patients. Based on these observations, 1α-hydroxylated vitamin D compounds have been proposed as therapeutic agents for the treatment of leukemoid diseases (Suda et al., U.S. Patent No. 4,391,802).

Although these known 1α-hydroxyvitamin D compounds tested by Suda et al. (supra), namely 1α-hydroxyvitamin D_3 (1α-OH-D_3) and 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$), are indeed highly effective in causing differentiation of leukemic cells, a serious disadvantage to their use as antileukemic agents is the inherent, and hence unavoidable high calcemic activity of these substances. Thus, 1α,25-$(OH)_2D_3$, the most potent vitamin-derived antileukemic agent known thus far, is also the most potent calcemic agent, and the antileukemic potency of 1α-OH-D_3 is likewise correlated with high calcemic activity. The administration of these compounds, at the dosage level where they are effective as antileukemic drugs (e.g. 1 μg/day as specified in the examples of the Suda et al. patent), would necessarily produce elevated, potentially excessive, calcium levels with attendant serious medical complications, particularly in patients already suffering from debilitating disease. Because of the high intrinsic potency of the known

1α-hydroxyvitamin D compounds in raising calcium levels, their use as antileukemic agents may be precluded.

A preferred method of treatment of viral diseases clearly would be the administration of compounds characterized by a high antileukemic to calcemic activity ratio, that is, of compounds exhibiting an enhanced potency in causing differentiation of leukemic cells as compared to their potency in raising serum calcium levels.

The compounds of this invention are also preferentially active in inducing the differentiation of malignant cells to non-malignant cells, i.e. in antineoplastic activity as measured by leukemia cell differentiation, while being no more active than 1α,25-dihydroxyvitamin D_3 in their effect on calcium metabolism. Because of this unique and unexpected combination of properties, the novel side-chain homovitamin D compounds of this invention represent superior and preferred agents for the treatment of leukemias, and viral diseases, such as AIDS.

When administered to human promyelocytic leukemia cells (HL-60 cells) grown in culture, the side-chain homovitamin D compounds of this invention induce the differentiation of these cells to macrophages (monocytes). In several standard assays for measuring differentiation activity, these compounds were shown to be more effective than 1α,25-$(OH)_2D_3$, the most active vitamin D derivative known thus far.

The extent of differentiation induced by the tested vitamin D derivatives was expressed as the percentage of cells that exhibit functional and enzymatic markers characteristic of monocytes. The two markers assayed were a) the ability of the cells to phagocytize dead yeast, and b) the ability of the cells to produce superoxide (reduce nitro-tetrazalium blue) when stimulated with phorbol esters.

This "% phagocytic cells" indicates the percent of differentiation induced by the test compounds. Results are summarized in Table 9 below.

Table 9

Percent phagocytic (differentiated) cells produced in HL-60 cell cultures treated with vitamin D compounds at various concentrations

Compound Administered	Concentration (moles/liter)						
	0[a,b]	$3x10^{-10}$	$5x10^{-10}$	$1x10^{-9}$[b]	$1x10^{-8}$[b]	$1x10^{-7}$[b]	$3x10^{-7}$
$1,25-(OH)_2D_3$	10±1.5	17	23	28±4	47±1	67±6	69
homo-cpd I*	10±1.5	28	38	44±5	72±2	76±3	77
homo-cpd II**	10±1.5	22	42	48±6	70±0	78±4	83

[a]Control level; cell cultures were treated with solvent ethanol only.

[b]Results tabulated in these columns represent the mean ± SEM of three different experiments, each done in duplicate.

*1α,25-dihydroxy-26-homovitamin D_3

**1α,25-dihydroxy-22E-dehydro-26-homovitamin D_3

The results in Table 9 show that the homo compounds are significantly more potent than 1,25-$(OH)_2D_3$. At all concentrations, the homo compounds achieve a greater degree of differentiation of the leukemia cells than 1α,25-$(OH)_2D_3$, the most active compound known thus far. For example, at a concentration of 10^{-8} molar the homo compounds achieve a differentiation of 70%, whereas 1,25-$(OH)_2D_3$ at the same concentration gives only about 47% differentiated cells. To achieve 50% differentiation requires a concentration of 1 x 10^{-9}M of the homo compounds, but about 1 x 10^{-8}M of 1α,25-$(OH)_2D_3$, i.e. a difference in potence of about 10-fold. The results of the NBT assay are shown in Table 10 below.

Table 10

Percent of cells in HL-60 cell cultures exhibiting nitroblue tetrazolium (NBT) reduction activity after treatment with Vitamin D Compounds at various concentrations

Compound Administered	Concentration (moles/liter)						
	0[a,b]	$3x10^{-10}$	$5x10^{-10}$	$1x10^{-9}$[b]	$1x10^{-8}$[b]	$1x10^{-7}$[b]	$3x10^{-7}$
$1,25-(OH)_2D_3$	10±1.5	15	27	31±4	45±4	69±7	65
homo-cpd I*	10±1.5	27	41	47±7	72±5	79±2	78
homo-cpd II**	10±1.5	22	44	49±4	70±2	79±5	80

[a] Control level; cell cultures treated with solvent ethanol only.

[b] Data represent the mean ± SEM of three separate experiments, each assayed in duplicate.

*1α,25-dihydroxy-26-homovitamin D_3

**1α,25-dihydroxy-22E-dehydro-26-homovitamin D_3

The results shown in Table 10 again establish that the homo compounds tested are more active than 1α,25-$(OH)_2D_3$ in inducing the differentiation of human mycloid leukemia cells to normal cells, in vitro. To achieve 60% differentiation of the leukemic cells as measured by this NBT reduction assay, requires a concentration of 2×10^{-9}M of the homo compounds; to achieve the same degree of differentiation with 1α,25-$(OH)_2D_3$ requires a concentration of 3.5×10^{-8}M--a 17-fold difference in potency.

Thus, both of the above assays confirm the high potency of the homovitamin D compounds in inducing the differentiation of leukemic cells. In addition, the above results show that in this differentiation activity these homovitamin D compounds are significantly more potent than 1α,25-$(OH)_2D_3$.

Since this differentiating activity is expressed in the case of human leukemia cells (HL-60), it is clear that these novel homovitamin D compounds can be used effectively against leukemias in human subjects. At the same time, these compounds do not exhibit enhanced calcemic activity, but are about as active as 1α,25-$(OH)_2D_3$. Thus, these homovitamin D compounds are characterized by a high antineoplastic to calcemic activity ratio. By virtue of this novel and desirable biological property, these side-chain homo compounds would function as superior thereapeutic agents for the treatment of AIDS.

For the treatment of human leukemia or AIDS, the homovitamin D compounds of this invention are administered to subjects in dosages sufficient to induce the differentiation of myeloid cells to macrophages. Suitable dosage amounts are as described above, it being understood that dosages can be adjusted according to the severity of the disease or the response or the condition of subject as is well-understood in the art.

Biological Activity of Cyclopentano Vitamin D Analogs

The vitamin D analogs, cyclopentano-1,25-dihydroxy-vitamin D_3 and cyclopentano-1,25-dihydroxy-22E-dehydro-vitamin D_3 were assayed for both calcemic activity and differentiation activity, using established procedures known in the art. The assay procedures and results obtained are described in the following examples.

Intestinal calcium transport activity and bone calcium mobilization activity of compounds.

Male weanling rats (obtained from Harlan-Sprague Dawley Co., Madison, WI) were fed a low calcium, vitamin D-deficient diet (0.22% Ca, 0.3% P) as described by Suda et al. (J. Nutr. 100, 1049-1052, 1970), for a total of 4 weeks ad libitum. At the end of the third week, the animals were divided randomly into groups of 6 rats each. One group (the control group) received a daily dose of solvent vehicle (0.1 mL of 95% propylene glycol/5% ethanol) by interperitoneal (i.p.) injection for a total of 7 days. The other groups received the amounts of test compound (i.e. 1,25-$(OH)_2D_3$, compound I, or compound II) as indicated in Table ii, dissolved in the same amount of solvent vehicle by daily injection over a period of 7 days. The animals were killed 24 hours after the last injection, their intestines were removed for intestinal calcium transport measurements, and their blood was collected for the assay of bone calcium mobilization (measurement of serum calcium levels). Intestinal calcium transport was measured by the everted gut sac technique [Martin & DeLuca, Am. J. Physiol. 216, 1351 (1969)] as described by Halloran and DeLuca [Arch. Biochem. Biophys. 208, 477-486 (1981)]. The results, expressed in the usual fashion as a ratio of serosal/mucosal calcium concentrations, are given in Table 11 below. Bone calcium mobilization was assayed by measuring serum calcium levels, using the standard procedures: 0.1 mL aliquots of serum were diluted with 1.9 mL of a 0.1% aqueous solution of $LaCl_3$ and calcium concentrations were then determined directly by atomic absorption spectroscopy. Results, expressed as mg % calcium, are also presented in Table 11 below.

Table 11

Intestinal Calcium Transport and Bone Calcium Mobilization (Serum Calcium Levels) Activity of the Cyclopentano-Vitamin D Analogs

Compound Administered	Amount ng/day	Ca Transport [Ca serosal]/ [Ca mucosal]	Serum Calcium mg%
		mean ± S.E.M.	mean ± S.E.M.
none (control)	0	2.4 ± 0.22	3.7 ± 0.06
$1,25-(OH)_2D_3$	50	8.3 ± 0.43	4.6 ± 0.10
Cyclopentano-1,25-(OH) D	25	7.7 ± 0.37	5.5 ± 0.31
	125	10.4 ± 0.10	7.4 ± 0.06
Cyclopentano-$1,25-(OH)_2$-22-dehydro-D	50	8.3 ± 0.81	5.9 ± 0.14

Differentiation activity of Cyclopentano Compounds.

Degree of differentiation of HL-60 cells (human leukemia cells) in response to test compounds was assessed by three different assays: NBT reduction, esterase activity, and phagocytosis activity. The NBT reduction and phagocytosis assays were carried out as described by DeLuca et al. in U.S.

Patent 4,717,721. The third assay, measuring nonspecific acid esterase as a marker for degree of differentiation was conducted according to the method given in Sigma Kit No. 90, available from Sigma Chemical Corp., St. Louis, MO [see also, Ostrem et al., Proc. Natl. Acad. Sci. USA 84, 2610 (1987); Ostrem et al., J. Biol. Chem. 262, 14164 (1987)]. Results are shown in Table 12 below. The data for the three assays are presented as the percent of differentiated cells resulting from treatment with various concentrations of 1,25-$(OH)_2D_3$ (used as comparison standard) or the cyclopentano-vitamin D analogs.

Table 12

Differentiation Activity of Cyclopentano-1,25-$(OH)_2D_3$ and Cyclopentano-1,25-$(OH)_2$-22-dehydro-D_3 in HL-60 Cell Cultures

Compound Administered	Concentration (molar)	% Differentiated Cells		
		NBT	Esterase	Phago-cytosis
1,25-$(OH)_2D_3$	1×10^{-7}	89 ± 3	93 ± 2	88 ± 3
	1×10^{-8}	58 ± 4	63 ± 4	59 ± 3
	1×10^{-9}	34 ± 3	37 ± 3	34 ± 2
Cyclopentano-1,25-$(OH)_2D_3$	5×10^{-8}	90 ± 4	88 ± 4	86 ± 3
	1×10^{-8}	81 ± 3	80 ± 4	82 ± 4
	1×10^{-9}	63 ± 2	66 ± 4	65 ± 4
	5×10^{-9}	46 ± 2	45 ± 3	45 ± 3
Cyclopentano-1,25-$(OH)_2$-22-dehydro-D_3	1×10^{-7}	95 ± 3	95 ± 3	92 ± 6
	5×10^{-8}	90 ± 3	91 ± 2	89 ± 3
	1×10^{-8}	80 ± 3	76 ± 4	78 ± 4
	5×10^{-9}	63 ± 2	67 ± 4	63 ± 3
	1×10^{-9}	47 ± 3	45 ± 2	49 ± 3
	5×10^{-10}	39 ± 3	39 ± 3	40 ± 3

The preceding test results establish that the new cyclopentano analogs, possess high calcemic and differentiation activity. Indeed, the assay results listed in Table 11 and Table 12 show that, with respect to calcemic activity and differentiation activity, the two cyclopentano

vitamin D analogs are more potent than the natural hormone, 1,25-$(OH)_2D_3$. Thus, the calcium transport response elicited by the cyclopentano analogs (see Table 11) is approximately the same as that given by 1,25-$(OH)_2D_3$ in their effect on calcium mobilization from bone (Table 11). Similarly, the data in Table 12 show that the cyclopentano analogs are approximately five times more active than 1,25-$(OH)_2D_3$ in inducing the differentiation of leukemic cells. This is evident, for example, from the entries showing that both cyclopentano compounds achieve 90% differentiation at a concentration of 5×10^{-8}M, whereas a five-fold higher concentration (1×10^{-7}M) of 1,25-$(OH)_2D_3$ is required to produce the same degree of differentiation.

Based on these results, one can conclude that both of the cyclopentano analogs can be used effectively as calcium regulating agents or as differentiation-inducing agents. Thus, the new analogs can be employed in the prophylaxis or treatment of calcium metabolism disorders such as renal osteodystrophy, vitamin D-resistant rickets, osteoporosis and related diseases. Likewise, their high potency in inducing the differentiation of analogs can be used in place of such known compounds as 1,25-$(OH)_2D_3$ for the treatment of neoplastic disease, especially leukemias, and now AIDS.

Biological Activity of Other Vitamin D Analogs:

1,25-Dihydroxyvitamin D_3, the hormonal form of vitamin D, induces differentiation of HL-60 human promyelocytes into monocyte-like cells in vitro. The relative activity of 30 analogs of 1,25-dihydroxyvitamin D_3 in inducing development of monocytic markers in HL-60 cells was assessed. The three differentiation markers assayed were nonspecific acid esterase activity, nitroblue tetrazolium reducing activity, and phagocytic capacity.

Activity Ratio (AR) -- The data from each assay was used to construct 3 log dose-response curves for each analog. The ED_{50}, i.e. the concentration

required to achieve 50% differentiation after 4 days, was obtained directly from each curve. An average ED_{50} was calculated from the 3 assays for each analog. Relative activity (AR) is the ratio of the average ED_{50} of the analog to the ED_{50} of $1,25\text{-}(OH)_2D_3$ (i.e. 10^{-8} M). The AR value, therefore, expresses the potency of an analog to induce maturation of HL-60 cells under the above mentioned conditions, relative to that of the natural hormone.

Figure 6 shows the structures of most of the analogs studied. Figures 7 through 11 are representative log dose-response curves for the various analogs. Steroidal side-chain structures are shown above each curve, and each figure provides the curves for one of the assays used. Similar curves were prepared for the other 2 assays and were used to calculate the ED_{50}s for each analog. Table 13 provides the ED_{50}s determined by each of the 3 assays for most of the analogs as well as the calculated activity ratios (AR).

Figure 7 shows the activity of five 1α-hydroxylated analogs that do not contain side-chain hydroxyl groups. 1α-OH-D_3 (1j) is 100 times less active than $1,25\text{-}(OH)_2D_3$ (1a) (ED_{50}s=10^{-6} M and 10^{-8} M, respectively; Table 13), indicating that loss of the 25-hydroxyl group diminishes potency by two orders of magnitude. In contrast, methylation of the 25-hydroxy group to produce the methyl ether of the natural hormone (1n) results in only a 6-7-fold decrease in activity. Introduction of a Δ^{22}-trans double bond improves the activity of 1-OH-D_3 2-fold: Δ^{22}-trans-1-OH-D_3 (1k) induces 50% differentiation at 5×10^{-7} M, compared to the 10^{-6} M required for the saturated 1-OH-D_3. Isomerization of the Δ^{22}-trans to Δ^{22}-cis (1m) as well as epimerization of the 1α-OH to 1β-OH practically eliminates all activity: Even at 10^{-6} M, these compounds induce only 10-20% differentiation.

Figure 8 compares the activity of a series of 25-OH analogs having no 1α-hydroxyl group (2a-2f). As s en with 25-OH-D_3 (2a), loss of the 1α-OH leads to an 80-fold reduction in activity. 25-OH-D_2 (2b) and 24-epi-25-OH-D_2 (2c) can induce 50% differentiation at 4 x 10^{-7} M and 3 x 10^{-7} M, respectively, being approximately 2-fold more active than 25-OH-D_3. This observation agrees with the result in Figure 7 that introduction of a trans double bond at C-22 improves the activity 2-fold. Both 25-OH-D_2 isomers have approximately the same activity in this system indicating a tolerance for either R- or S-methyl stereochemistry at C-24. Introduction of fluorine groups in the side chain as in 26,27-F_6-25-OH-D_2 (2e) improves the activity of 25-OH-D_2 two-fold. This agrees with previous observations of Shiina et al. and Koeffler et al. that fluorination either in the 24-position or 26,27-position improves ability of 1,25-$(OH)_2D_3$ to induce myeloid cell maturation 4 to 7-fold. Isomerization of the side-chain double bond from Δ^{22} to Δ^{23}-position (2d) which creates an sp2 planar center at C-24 decreases the activity of 25-OH-D_2 two-fold. 24R,25-$(OH)_2D_3$ (2f) is less active than 25-OH-D_3, suggesting that the 24-hydroxyl group in the presence of a 25-hydroxyl function slightly reduces activity in this system. Similarly, 24-hydroxylation of 1,25-$(OH)_2D_3$ reduces its activity in HL-60 cells.

The effect of side chain elongation and truncation as well as the effect of isomerization of the triene system from 5,6-cis to 5,6-trans are examined in Figure 9. Generally, elongation by one carbon improves the activity of the natural hormone by one order of magnitude, while truncation of the side-chain by each carbon removed diminishes activity by one order of magnitude. 24-Homo-1,25-$(OH)_2D_3$ (1b) and 26-homo-1,25-$(OH)_2D_3$ (1c) are 8-fold more active than the natural hormone since they can induce 50% maturation of the HL-60 cells at 1.3 x 10^{-9} M compared to the 10^{-8} M required for 1,25-$(OH)_2D_3$.

Introduction of unsaturation at C-22 resulted in analogs that retained the 8-fold improved potency These results cannot be explained on the basis of the affinity of the homoanalogs for the 1,25-$(OH)_2D_3$ receptor. Competition studies show these compounds to have equivalent ability with 1,25-$(OH)_2D_3$ in displacing the natural hormone from its binding site in chick and rat intestine as well as HL-60 cells Deletion of one carbon (24-nor-1,25-$(OH)_2D_3$) (1d) or two carbons (23,24-nor-1,25-$(OH)_2D_3$) (1e) from the steroid side-chain results in a 13- and 220-fold reduction in activity, respectively. The binding affinity of these truncated analogs, as determined by displacement studies using the chick intestinal 1,25-$(OH)_2D_3$ receptor, closely parallels their activity in the HL-60 system: 1d and 1e have 10-fold and 160-fold lower affinity, respectively, for the 1,25-$(OH)_2D_3$ receptor (S. Lee, H. K. Schnoes, and H. F. DeLuca, unpublished results).

A derivative with a 5,6-trans-triene modification is only 7 times less effective than the 5,6-cis compound (Figure 9). Thus, 7×10^{-7} M concentration of the 5,6-trans isomer of 24-nor-1,25-$(OH)_2D_3$ is required to achieve 50% differentiation compared to 1.3×10^{-7} M needed of the 5,6-cis derivative (1d). Recognizing the importance of the 1α-hydroxy group for effectiveness in the HL-60 system, this relative high activity of the 5,6-trans derivative is probably a result of the transposition of the 3β-hydroxy group into a pseudo-1α-hydroxy position. Similarly, 25-OH-6,19-epoxyvitamin D_3, which lacks an 1α-hydroxyl, shows unexpected high activity in the HL-60 system

Figure 10 presents the activity of primary alcohol side chains of various lengths as well as short chain analogs (compounds 1o-1v). 26,27-bis-nor-1,25-$(OH)_2D_3$ (1o) differs from the natural hormone only in its lack of the two methyl groups flanking the 25-hydroxy substituent. Yet, this compound is two orders of magnitude less effective than 1,25-$(OH)_2D_3$. Sequential deletion of

ne carbon fr m the side chain of 1o represented by analogs 1p-1r has no furth r effect in decreasing the activity: 23,24,25,26,27-pentanor-1,22-$(OH)_2D_3$ (1r) is also two orders of magnitude less effective than 1,25-$(OH)_2D_3$. Oxidation of the C-22-hydroxyl to a less bulky and less polar aldehyde (1s) improves the activity two-fold; AR is reduced from 170 to 80-fold lower than the natural hormone. Removal of the oxygen substituent from C-22 results in a dramatic improvement in activity: 1α-OH-bishomopregna- (1t) and 1α-OH-homopregnacholecalciferol (1u) are only 20-fold less active than 1,25-$(OH)_2D_3$. In view of the fact that 1α-OH-D_3, an analog that has lost the 25-hydroxyl substituent while retaining the original length of the steroidal side chain is 100-fold less active than 1,25-$(OH)_2D_3$, these results are remarkable. The high activity of 1t and 1u can be explained in terms of their surprising high affinity for the 1,25-$(OH)_2D_3$ receptor. 1α-OH-homopregnacholecalciferol and 1α-OH-bishomopregnacholecalciferol are only 4- and 11-fold less effective than 1,25-$(OH)_2D_3$ in displacing the natural ligand from its binding site on the chick intestinal receptor (22).

1,24R-$(OH)_2D_3$ (1f) has equivalent activity with the natural hormone, while its stereoisomer, 1,24S-$(OH)_2D_3$ (1g) is half as active (Table 13). In vivo, 1,24S-$(OH)_2D_3$ is less active in stimulating calcium transport and bone calcium mobilization and has equal affinity for the receptor compared to 1,25-$(OH)_2D_3$ Matsui et al. have also shown that 1,24R-$(OH)_2D_3$ shows the same potency as 1,25-$(OH)_2D_3$ in inducing monocyte/granulocyte associated plasma membrane antigens, and 1,24S-$(OH)_2D_3$ is only slightly less active. This indicates a small discrimination against the 24S- stereoisomer which could be due to a steric effect of the C-24S-substituent since 1,25-$(OH)_2D_2$ (1i) that has a methyl group in the C-24S-position is also 2-fold less active than 1,25-$(OH)_2D_3$ (Table 13). In fact, 1,25-$(OH)_2D_2$ (1i) and 1,24R,25-$(OH)_3D_3$ (1h)

uniquely produce unexpected and highly reproducible biphasic log dose-response curves (Figure 11) with 2 ED_{50}s at 2-3 x 10^{-8}M and 9 x 10^{-8}M.

In summary, of the known metabolites of vitamin D, 1,25-dihydroxyvitamin D_3 is the most active; fifty percent of the cells exhibit the mature phenotype following a 4-day treatment with 10^{-8}M 1,25-dihydroxyvitamin D_3. Removal of either the C-1 or C-25-hydroxyl group reduces activity by two orders of magnitude, while epimerization of the 1α- to 1β-hydroxyl group virtually abolishes activity. Elongation of the steroidal side chain of 1,25-dihydroxyvitamin D_3 by addition of one carbon at C-24 or C-26 improves the potency by an order of magnitude. Truncation of the steroidal side chain leads to a ten-fold reduction in activity for each carbon removed. Elimination of the C-26 and C-27 methylk groups reduces activity 100-fold. Analogs with short aliphatic side chains as 1α-hydroxy-homo- and bishomopregnacholecalciferol have surprisingly high activity, being only 20-fold less potent than the natural hormone. The activity of most analogs in the HL-60 system parallels their known relative affinities for the well characterized 1,25-dihydroxyvitamin D_3 receptor in chick intestine, providing further evidence that this function of 1,25-dihydroxyvitamin D_3 is receptor-mediated.

It should be specifically noted that 1α-hydroxyvitamin D_3 is less than 100 times as active as 1α,25-dihydroxyvitamin D_3 (see Table 13) in causing differentiation of HL60 cells <u>in vitro</u>. However, <u>in vivo</u> it is well established that 1α-hydroxyvitamin D_3 is rapidly converted to 1α,25-dihydroxyvitamin D_3, Hollick et al, <u>Science</u>, Vol. 190, pages 576-578 (1975) and Hollick et al, <u>Journal of Clinical Endocrinology & Metabolism</u>, Vol. 44, pages 595-598 (1977), which compound as shown herein is highly potent in cell differentiation. Thus, it is clear that the human body can rapidly convert

the relatively inactive 1α-hydroxylated vitamin D compounds to metabolites highly active in causing cell differentiation. This in vivo capability makes possible the treatment of malignancies and viral diseases such as AIDS with 1α-hydroxylated vitamin D compounds that do not initially have a hydroxyl group at the 24 or 25 carbon position in the side chain.

In addition, the present invention provides compositions and methods for treating lentivirus infections, and attendant immune and infectious disorders. With respect to lentiviruses, this is accomplished by administering an effective amount of a vitamin D compound which compound when tested in vitro is capable of inhibiting the replication of the lentivirus. Lentiviruses are well known, and in general terms can be described as retro viruses having a relatively slow pathology with a genetic structure common to this group of viruses. For example, lentiviruses include human immunodeficiency virus type 1 (HIV-1), human immunodeficiency virus type 2 (HIV-2), simian immunodeficiency virus (SIV), equine encephalitis-arthritis virus (CAEV), visna-naedi virus, bovine leukosis virus (BLV), and feline immunodeficiency virus (FIV).

TABLE 13

Relative activities of 1,25-$(OH)_2D$ analogs in inducing HL-60 differentiation

Compound	ED_{50} Nitrobule Tetrazolium Reduction (M)	ED_{50} Phagocytic activity (M)	ED_{50} Nonspecific Acid Esterase Activity (M)	Activity Ratio (AR)
1a	1.0×10^{-8}	1.0×10^{-8}	1.1×10^{-8}	1
1b	1.2×10^{-9}	1.6×10^{-9}	1.3×10^{-9}	0.13
1c	1.2×10^{-9}	1.6×10^{-9}	1.3×10^{-9}	0.13
1d	1.5×10^{-7}	1.2×10^{-7}	1.6×10^{-7}	13
1e	2.4×10^{-6}	2.0×10^{-6}	2.1×10^{-6}	220
1f	8.0×10^{-9}	7.0×10^{-9}	1.2×10^{-8}	1
1g	3.0×10^{-8}	2.7×10^{-8}	1.7×10^{-8}	2
*1h	3.5×10^{-8}	3.5×10^{-8}	3.5×10^{-8}	4
*1i	2.0×10^{-8}	2.0×10^{-8}	2.6×10^{-8}	2
1j	9.3×10^{-7}	1.0×10^{-6}	1.0×10^{-6}	100
1k	4.7×10^{-7}	6.0×10^{-7}	4.0×10^{-7}	50
1l	5.8×10^{-7}	5.8×10^{-7}	6.0×10^{-7}	60
1m	$>>10^{-6}$	$>>10^{-6}$	$>>10^{-6}$	>>100
1n	6.5×10^{-8}	6.5×10^{-8}	6.5×10^{-8}	7
1o	1.0×10^{-6}	1.2×10^{-6}	1.0×10^{-6}	100
1p	7.0×10^{-7}	6.0×10^{-7}	1.0×10^{-6}	80
1q	3.0×10^{-6}	2.0×10^{-6}	2.0×10^{-6}	220
1r	1.4×10^{-6}	1.8×10^{-6}	2.0×10^{-6}	170
1s	7.5×10^{-7}	8.2×10^{-7}	8.5×10^{-7}	80
1t	2.3×10^{-7}	2.5×10^{-7}	2.7×10^{-7}	25
1u	1.8×10^{-7}	1.9×10^{-7}	1.7×10^{-7}	18
1v	$>>10^{-6}$	$>>10^{-6}$	$>>10^{-6}$	>>100

-continued-

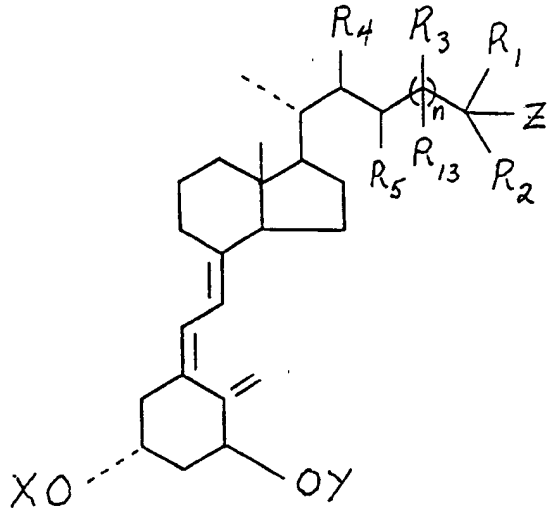
Compound	ED_{50} Nitrobule Tetrazolium Reduction (M)	ED_{50} Phagocytic activity (M)	ED_{50} Nonspecific Acid Esterase Activity (M)	Activity Ratio (AR)
2a	8.4×10^{-7}	8.0 x 10-7	8.0×10^{-7}	80
2b	3.8×10^{-7}	4.6×10^{-7}	3.4×10^{-7}	40
2c	3.0×10^{-7}	2.8×10^{-7}	2.8×10^{-7}	30
2d	7.5×10^{-7}	8.0×10^{-7}	8.6×10^{-7}	80
2e	1.3×10^{-7}	1.2×10^{-7}	1.3×10^{-7}	13
2f	1.1×10^{-6}	9.5×10^{-7}	9.5×10^{-7}	100

* These compounds gave a biphasic response. The values represent the ED_{50} derived from the dose response curve at the lower concentration of analog.

HL-60 cells were cultured for four days in the presence of the indicated concentration of 1,25-$(OH)_2D_3$ analogs. The analog concentration capable of inducing 50% maturation by the three assays was derived from log dose-response curves. AR is the ratio of the analog average ED_{50} to the ED_{50} for 1,25-$(OH)_2D_3$ (10^{-8} M) and relates the activity of the analog to that of the natural hormone. Untreated cultures consistently show 5-7% monocytic cells by the above three assays.

We claim:

1. A method for treating human immunodeficiency virus infection and acquired immune deficiency syndrome which comprises administering to a patient an effective amount of a compound of the formula:



where R_4 and R_5 represent hydrogen, deuterium, or when taken together R_4 and R_5 represent a carbon-carbon double bond or a carbon-carbon triple bond, R_{13} represents hydrogen, hydroxy, protected hydroxy, fluorine, deuterium or an alkyl group, Z represents hydrogen, hydroxy, protected-hydroxy, R_3 represents hydrogen, deuterium, hydroxy, protected-hydroxy, fluorine or an alkyl group, X and Y which may be the same or different, are hydrogen or a hydroxy-protecting group, R_1 represents the group $-CF_3$, $-CD_3$, or $-(CH_2)_q-H$ and R_2 represents the group $-CF_3$, $-CD_3$, or $-(CH_2)_p-H$, and where n, q and p are integers having independently the values of 1 to 5, and R_1 and R_2 when taken together represent the group $-(CH_2)_m-$ where m is an integer having the value of 2 to 5.

2. The method of claim 1 wherein said compound is 1α,25-dihydroxy-vitamin D_3.

3. The method of claim 1 wherein said compound is 1α,25-dihydroxy-24,24-difluoro-vitamin D_3.

4. The method of claim 1 wherein said compound is 1α,25-dihydroxy-26,27-hexadeutero-vitamin D_3.

5. The method of claim 1 wherein said compound is 1α,25-dihydroxy-26,27-hexafluorovitamin D_3.

6. The method of claim 1 wherein said compound is 1α-hydroxyvitamin D_3.

7. The method of claim 1 wherein the compound is 24-homo-1α,25-dihydroxy-22-dehydrovitamin D_3.

8. The method of claim 1 wherein the compound is 24-dihomo-1α,25-dihydroxy-22-dehydrovitamin D_3.

9. The method of claim 1 wherein the compound is 24-trihomo-1α,25-dihydroxy-22-dehydrovitamin D_3.

10. The method of claim 1 wherein the compound is 26,27-dimethyl-24-dihomo-1α,25-dihydroxy-22-dehydrovitamin D_3.

11. The method of claim 1 wherein the compound is 26,27-dimethyl-24-trihomo-1α,25-dihydroxy-22-dehydrovitamin D_3.

12. The method of claim 1 wherein the compound is 26,27-diethyl-24-dihomo-1α,25-dihydroxy-22-dehydrovitamin D_3.

13. The method of claim 1 wherein the compound is 26,27-diethyl-24-trihomo-1α,25-dihydroxy-22-dehydrovitamin D_3.

14. The method of claim 1 wherein the compound is 26,27-dipropyl-24-dihomo-1α,25-dihydroxy-22-dehydrovitamin D_3.

15. The method of claim 1 wherein the compound is 26,27-dipropyl-24-trihomo-1α,25-dihydroxy-22-dehydrovitamin D_3.

16. The method of claim 1 wherein the compound is 24-homo-1α,25-dihydroxyvitamin D_3.

17. The method of claim 1 wherein the compound is 24-dihomo-1α,25-dihydroxyvitamin D_3.

18. The method of claim 1 wherein the compound is 24-trihomo-1α,25-dihydroxyvitamin D_3.

19. The method of claim 1 wherein the compound is 26,27-dimethyl-24-dihomo-1α,25-dihydroxyvitamin D_3.

20. The method of claim 1 wherein the compound is 26,27-dimethyl-24-trihomo-1α,25-dihydroxyvitamin D_3.

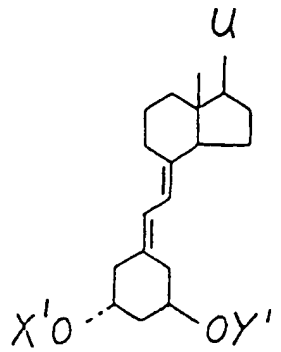
21. The method of claim 1 wherein the compound is 26,27-diethyl-24-dihomo-1α,25-dihydroxyvitamin D_3.

22. The method of claim 1 wherein the compound is 26,27-diethyl-24-trihomo-1α,25-dihydroxyvitamin D_3.

23. The method of claim 1 wherein the compound is 26,27-dipropyl-24-dihomo-1α,25-dihydroxyvitamin D_3.

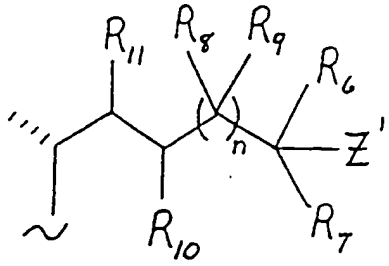
24. The method of claim 1 wherein the compound is 26,27-dipropyl-24-trihomo-1α,25-dihydroxyvitamin D_3.

25. A method for treating human immunodeficiency virus infection and acquired immune deficiency syndrome which comprises administering to a patient an effective amount of a compound of the formula:



where X^1 and Y^1 are each selected from the group consisting of hydrogen, acyl, alkylsilyl and alkoxyalkyl, and where U is selected from the group consisting of

alkyl, hydrogen, hydroxyalkyl, fluoroalkyl and a side chain of the formula



wherein Z^1 represents hydrogen, hydroxy or O-acyl, R_6 and R_7 are each selected from the group consisting of alkyl, deuteroalkyl, hydroxyalkyl and fluoroalkyl, or, when taken together represent the group -- $(CH_2)_m$ -- where m is an integer having a value of from 2 to 5, R_8 is selected from the group consisting of hydrogen, deuterium, hydroxy, fluorine, O-acyl, alkyl, hydroxyalkyl and fluoroalkyl, R_9 is selected from the group consisting of hydrogen, deuterium, fluorine, alkyl, hydroxyalkyl and fluoroalkyl, or, R_8 and R_9 taken together represent double-bonded oxygen or double-bonded carbon, R_{10} and R_{11} are each selected from the group consisting of hydrogen, deuterium, hydroxy, O-acyl, fluorine and alkyl, or, R_{10} and R_{11} taken together form a carbon-carbon double bond or a carbon-carbon triple bond, and wherein n is an integer having a value of from 1 to 5 and wherein the carbon at any one of positions 20, 22, or 23 in the side chain may be replaced by an O, S, or N atom.

26. The method of claim 25 wherein the compound is 1α,25-dihydroxy-19-nor-vitamin D_3.

27. The method of claim 25 wherein the compound is 1α-hydroxy-19-nor-vitamin D_3.

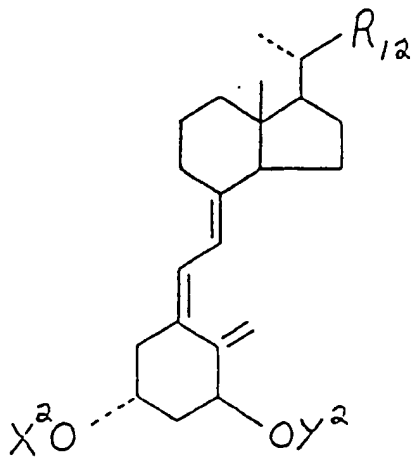
28. The method of claim 25 wherein the compound is 1α,25-dihydroxy-19-nor-vitamin D_2.

29. The method of claim 25 wherein the compound is 1α-hydroxy-19-nor-vitamin D_2.

30. The method of claim 25 wherein the compound is 1α-hydroxy-19-nor-24 epi-vitamin D_2.

31. The method of claim 25 wherein the compound is 1α,25-dihydroxy-19-nor-24 epi-vitamin D_2.

32. A method for treating human immunodeficiency virus infection and acquired immune deficiency syndrome which comprises administering to a patient an effective amount of a compound of the formula:



where R_{12} is hydrogen, methyl, ethyl or propyl and where each of X^2 and Y^2 represent, independently, hydrogen, an acyl group, or a hydroxy-protecting group.

33. The method of claim 32 wherein R_{12} is hydrogen.

34. The method of claim 32 wherein R_{12} is methyl.

35. The method of claim 32 wherein R_{12} is ethyl.

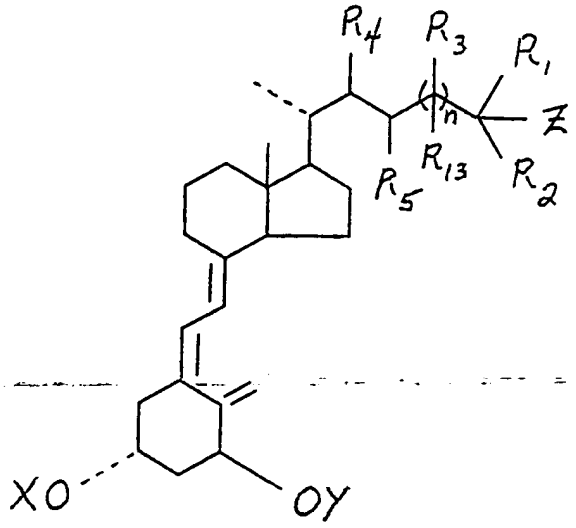
36. The method of claim 32 wherein R_{12} is propyl.

37. The method of claim 1 wherein said effective amount comprises about 0.01 μg/day to about 100 μg/day of said compound.

38. The method of claim 25 wherein said effective amount comprises about 0.01 μg/day to about 100 μg/day of said compound.

39. The method of claim 32 wherein said effective amount comprises about 0.01 μg/day to about 100 μg/day of said compound.

40. A composition for use in the treatment of human immunodeficiency virus infection and acquired immune deficiency syndrome which comprises an effective amount of a compound of the formula



where R_4 and R_5 represent hydrogen, deuterium, or when taken together R_4 and R_5 represent a carbon-carbon double bond or a carbon-carbon triple bond, R_{13} represents hydrogen, hydroxy, protected-hydroxy, fluorine, deuterium, or an alkyl group, Z represents hydrogen, hydroxy or protected-hydroxy, R_3 represents hydrogen, deuterium, hydroxy, protected-hydroxy, fluorine or an alkyl group, X and Y which may be the same or

different, are hydrogen or a hydroxy-protecting group, R_1 represents the group $-CF_3$, $-CD_3$, or $-(CH_2)_q-H$ and R_2 represents the group $-CF_3$, $-CD_3$, or $-(CH_2)_p-H$, and where n, q and p are integers having independently the values of 1 to 5, and R_1 and R_2 when taken together represent the group $-(CH_2)_m-$ where m is an integer having the value of 2 to 5; and a suitable carrier.

41. The composition of claim 40 wherein said compound is 1α,25-dihydroxy-vitamin D_3.

42. The composition of claim 40 wherein said compound is 1α,25-dihydroxy-24,24-difluoro-vitamin D_3.

43. The composition of claim 40 wherein said compound is 1α,25-dihydroxy-26,27-hexadeutero-vitamin D_3.

44. The composition of claim 40 wherein said compound is 1α,25-dihydroxy-26,27-hexafluorovitamin D_3.

45. The composition of claim 40 wherein said compound is 1α-hydroxyvitamin D_3.

46. The composition of claim 40 wherein the compound is 24-homo-1α,25-dihydroxy-22-dehydrovitamin D_3.

47. The composition of claim 40 wherein the compound is 24-dihomo-1α,25-dihydroxy-22-dehydrovitamin D_3.

48. The composition of claim 40 wherein the compound is 24-trihomo-1α,25-dihydroxy-22-dehydrovitamin D_3.

49. The composition of claim 40 wherein the compound is 26,27-dimethyl-24-dihomo-1α,25-dihydroxy-22-dehydrovitamin D_3.

50. The composition of claim 40 wherein the compound is 26,27-dimethyl-24-trihomo-1α,25-dihydroxy-22-dehydrovitamin D_3.

51. The composition of claim 40 wherein the compound is 26,27-diethyl-24-dihomo-1α,25-dihydroxy-22-dehydrovitamin D_3.

52. The composition of claim 40 wherein the compound is 26,27-diethyl-24-trihomo-1α,25-dihydroxy-22-dehydrovitamin D_3.

53. The composition of claim 40 wherein the compound is 26,27-dipropyl-24-dihomo-1α,25-dihydroxy-22-dehydrovitamin D_3.

54. The composition of claim 40 wherein the compound is 26,27-dipropyl-24-trihomo-1α,25-dihydroxy-22-dehydrovitamin D_3.

55. The composition of claim 40 wherein the compound is 24-homo-1α,25-dihydroxyvitamin D_3.

56. The composition of claim 40 wherein the compound is 24-dihomo-1α,25-dihydroxyvitamin D_3.

57. The composition of claim 40 wherein the compound is 24-trihomo-1α,25-dihydroxyvitamin D_3.

58. The composition of claim 40 wherein the compound is 26,27-dimethyl-24-dihomo-1α,25-dihydroxyvitamin D_3.

59. The composition of claim 40 wherein the compound is 26,27-dimethyl-24-trihomo-1α,25-dihydroxyvitamin D_3.

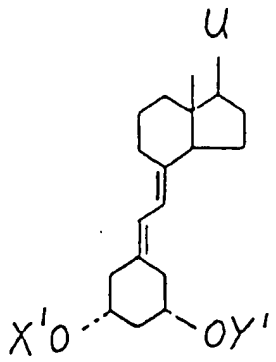
60. The composition of claim 40 wherein the compound is 26,27-diethyl-24-dihomo-1α,25-dihydroxyvitamin D_3.

61. The composition of claim 40 wherein the compound is 26,27-diethyl-24-trihomo-1α,25-dihydroxyvitamin D_3.

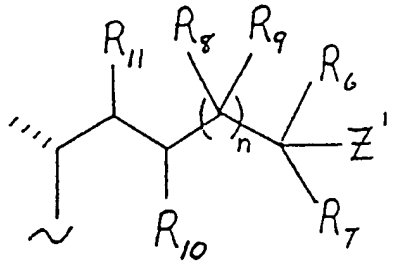
62. The composition of claim 40 wherein the compound is 26,27-dipropyl-24-dihomo-1α,25-dihydroxyvitamin D_3.

63. The composition of claim 40 wherein the compound is 26,27-dipropyl-24-trihomo-1α,25-dihydroxyvitamin D_3.

64. A composition for use in the treatment of human immunodeficiency virus infection and acquired immune deficiency syndrome which comprises an effective amount of a compound of the formula:



where X^1 and Y^1 are each selected from the group consisting of hydrogen, acyl, alkylsilyl and alkoxyalkyl, and where U is selected from the group consisting of alkyl, hydrogen, hydroxyalkyl, fluoroalkyl and a side chain of the formula:



wherein Z^1 represents hydrogen, hydroxy or O-acyl, R_6 and R_7 are each selected from the group consisting of alkyl, deuteroalkyl, hydroxyalkyl and fluoroalkyl, or, when taken together represent the group -- $(CH_2)_m$ -- where m is an integer having a value of from 2 to 5, R_8 is selected from the group consisting of hydrogen, deuterium, hydroxy, fluorine, O-acyl, alkyl, hydroxyalkyl and fluoroalkyl, R_9 is selected from the group consisting of hydrogen, deuterium, fluorine, alkyl, hydroxyalkyl and fluoroalkyl, or, R_8 and R_9 taken together represent double-bonded oxygen or double-bonded carbon, R_{10} and R_{11} are each selected from the group consisting of hydrogen, deuterium, hydroxy, O-acyl, fluorine and alkyl, or, R_{10}

and R_{11} taken together form a carbon-carbon double bond or a carbon-carbon triple bond, and wherein n is an integer having a value of from 1 to 5 and wherein the carbon at any one of positions 20, 22, or 23 in the side chain may be replaced by an O, S, or N atom; and a suitable carrier.

65. The composition of claim 64 wherein the compound is 1α,25-dihydroxy-19-nor-vitamin D_3.

66. The composition of claim 64 wherein the compound is 1α-hydroxy-19-nor-vitamin D_3.

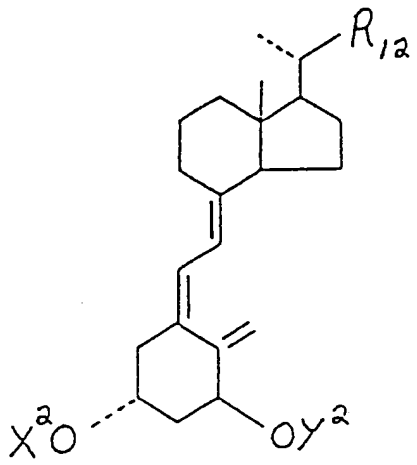
67. The composition of claim 64 wherein the compound is 1α,25-dihydroxy-19-nor-vitamin D_2.

68. The composition of claim 64 wherein the compound is 1α-hydroxy-19-nor-vitamin D_2.

69. The composition of claim 64 wherein the compound is 1α-hydroxy-19-nor-24 epi-vitamin D_2.

70. The composition of claim 64 wherein the compound is 1α,25-dihydroxy-19-nor-24 epi-vitamin D_2.

71. A composition for use in the treatment of human immunodeficiency virus infection and acquired immune deficiency syndrome which comprises an effective amount of a compound of the formula:



where R_{12} is hydrogen, methyl, ethyl or propyl and where each of X^2 and Y^2 represent, independently, hydrogen, an acyl group, or a hydroxy-protecting group; and a suitable carrier.

72. The composition of claim 71 wherein R_{12} is hydrogen.

73. The composition of claim 71 wherein R_{12} is methyl.

74. The composition of claim 71 wherein R_{12} is ethyl.

75. The composition of claim 71 wherein R_{12} is propyl.

76. The composition of claim 40 wherein said effective amount is between about 0.01 μg to about 100 μg per gram of the composition.

77. The composition of claim 64 wherein said effective amount is between about 0.01 μg to about 100 μg per gram of the composition.

78. The composition of claim 71 wherein said effective amount is between about 0.01 μg to about 100 μg per gram of the composition.

79. A method of treating human immunodeficiency virus infection and acquired immune deficiency syndrome which comprises administering to a patient an effective amount of a vitamin D compound which compound when tested in vitro is capable of stimulating the differentiation of a human cell line.

80. The method of claim 79 wherein said cell line is a U937 cell line.

81. The method of claim 79 wherein said cell line is a HL60 cell line.

82. The method of claim 79 wherein said cell line is a M1 cell line.

83. A method of treating human immunodeficiency virus infection and acquired immune deficiency syndrome which comprises administering to a patient an effective amount of a 1α-hydroxylated vitamin

D compound which compound upon administration to humans is converted to a metabolite and said metabolite in vitro will cause differentiation in a human cell line.

84. The method of claim 83 wherein said cell line is a U937 cell line.

85. The method of claim 83 wherein said cell line is a HL60 cell line.

86. The method of claim 83 wherein said cell line is a M1 cell line.

87. A method of treating lentivirus infections and attendant immune and infectious disorders which comprises administering to a patient an effective amount of a vitamin D compound which compound when tested in vitro is capable of inhibiting the replication of the lentivirus.

88. The method of claim 87 wherein said lentivirus is human immunodeficiency virus type 1.

89. The method of claim 87 wherein said lentivirus is human immunodeficiency virus type 2.

90. The method of claim 87 wherein said lentivirus is simian immunodeficiency virus.

91. the method of claim 87 wherein said lentivirus is equine infectious anemia virus.

92. The method of claim 87 wherein said lentivirus is caprine encephalitis-arthritis virus.

93. The method of claim 87 wherein said lentivirus is visna-naedi virus.

94. The method of claim 87 wherein said lentivirus is bovine leukosis virus.

95. The method of claim 87 wherein said lentivirus is feline immunodeficiency virus.

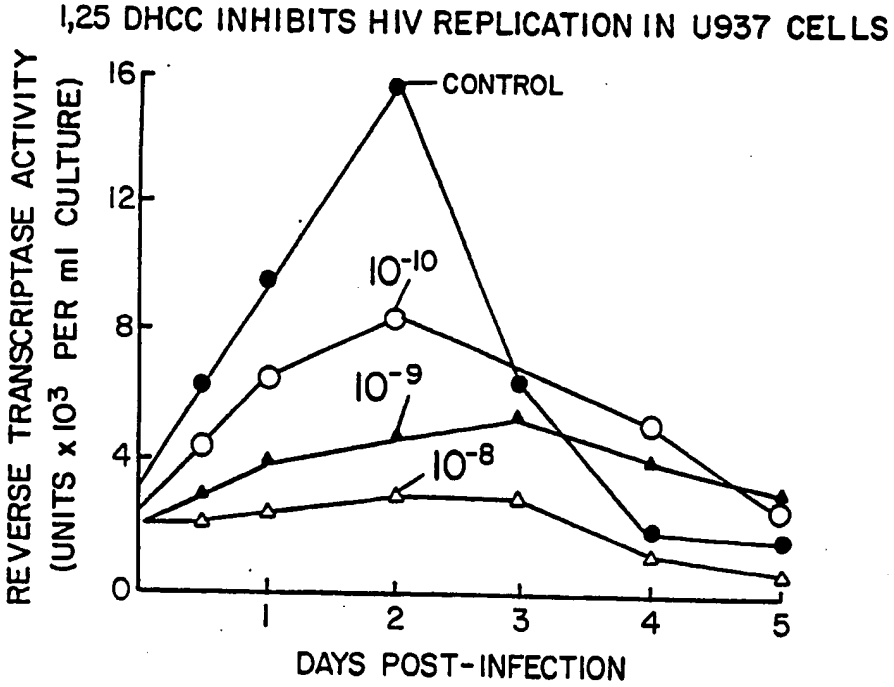

1,25 DHCC INHIBITS HIV REPLICATION IN U937 CELLS
REVERSE TRANSCRIPTASE ACTIVITY (UNITS x 10³ PER ml CULTURE)
16
12
8
4
0
CONTROL
10⁻¹⁰
10⁻⁹
10⁻⁸
1
2
3
4
5
DAYS POST-INFECTION

FIG. 1

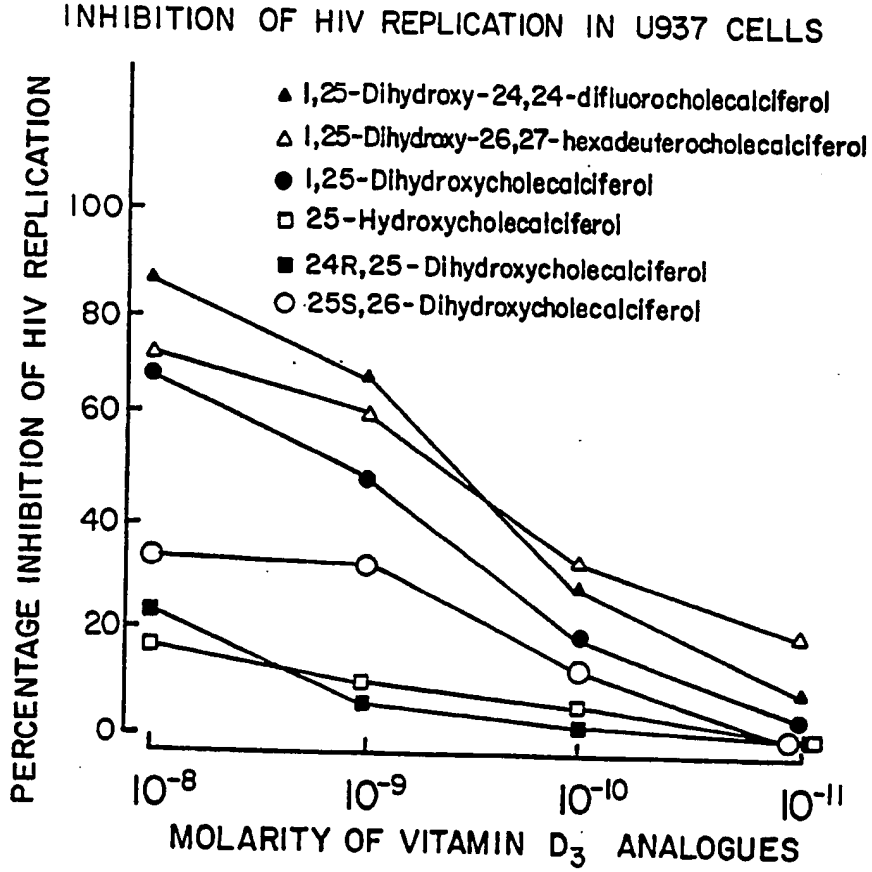
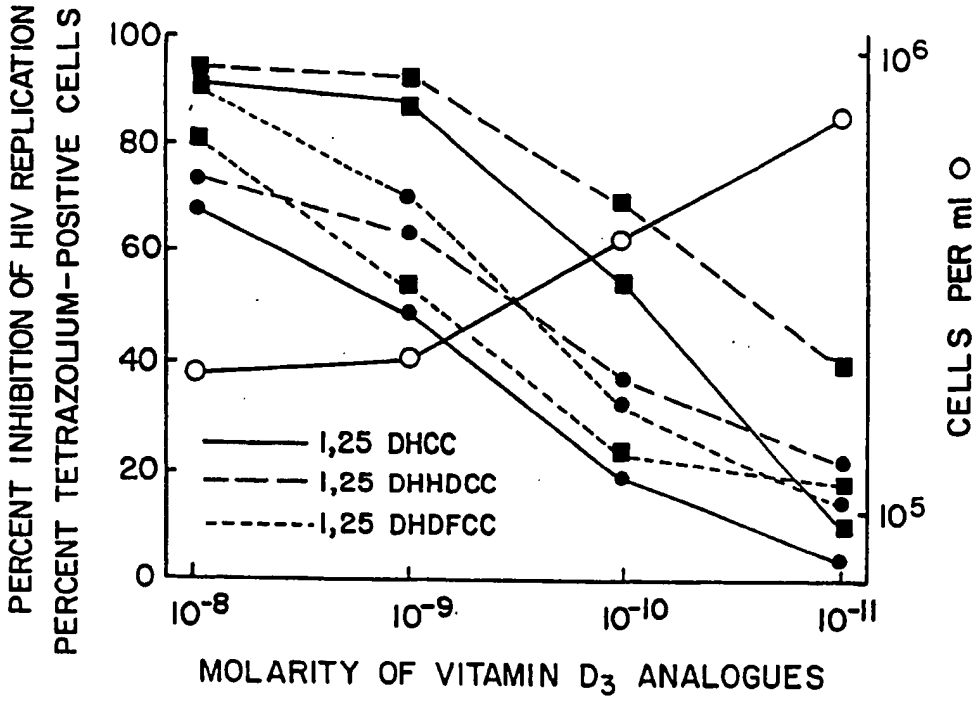

INHIBITION OF HIV REPLICATION IN U937 CELLS
PERCENTAGE INHIBITION OF HIV REPLICATION
100
80
60
40
20
0
▲ 1,25-Dihydroxy-24,24-difluorocholecalciferol
△ 1,25-Dihydroxy-26,27-hexadeuterocholecalciferol
● 1,25-Dihydroxycholecalciferol
□ 25-Hydroxycholecalciferol
■ 24R,25-Dihydroxycholecalciferol
○ 25S,26-Dihydroxycholecalciferol
10⁻⁸
10⁻⁹
10⁻¹⁰
10⁻¹¹
MOLARITY OF VITAMIN D₃ ANALOGUES

FIG. 2

FIG. 3 CELLULAR DIFFERENTIATION IS CORRELATED WITH HIV INHIBITION FOR VITAMIN D_3 ANALOGUE TREATMENT OF U937 CELLS



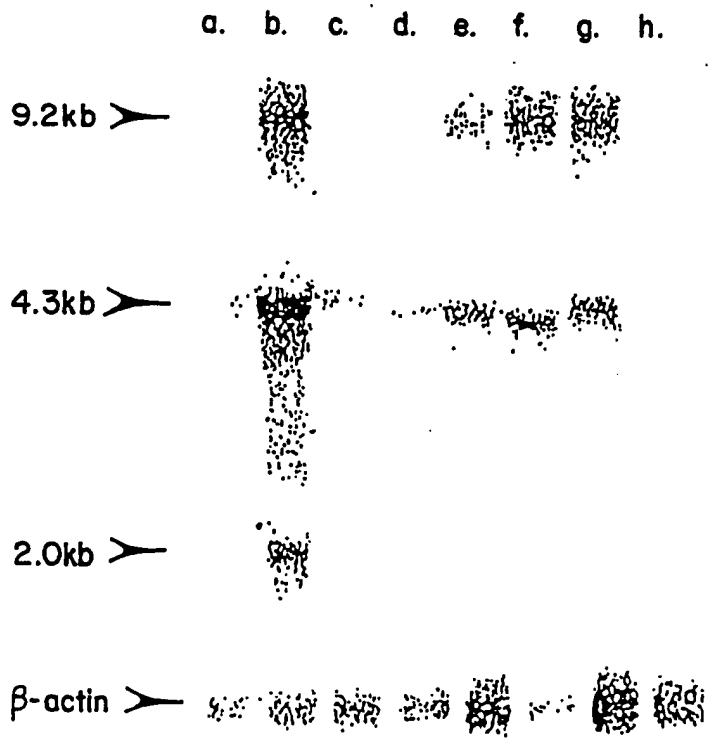


FIG. 4

SUBSTITUTE SHEET

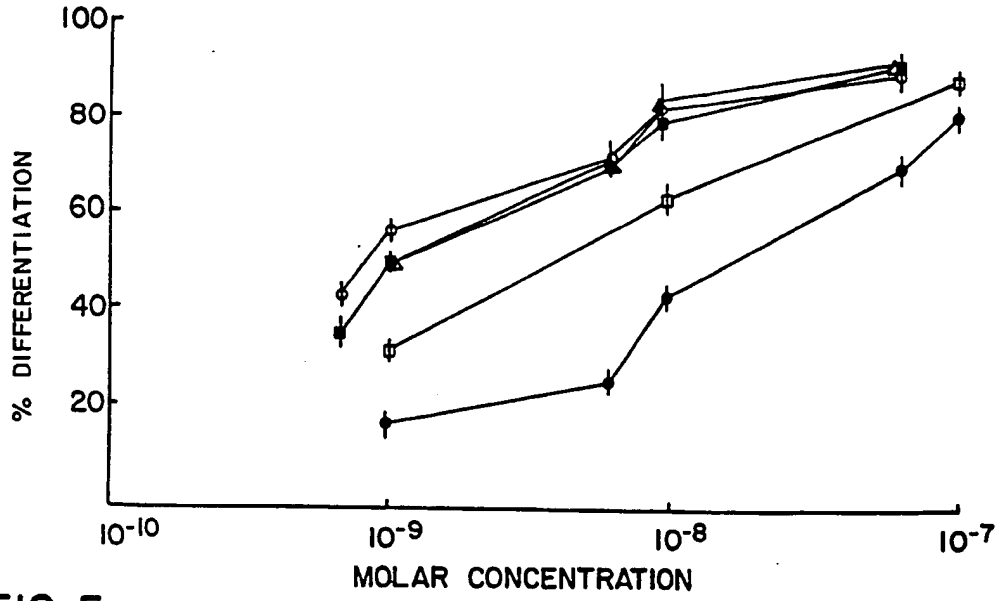


FIG. 5 DIFFERENTIATION ACTIVITY OF THE ANALOGUES OF 1,25-$(OH)_2D_3$. PERCENT DIFFERENTIATION IS COMPUTED ON THE BASIS OF CELLS SHOWING NONSPECIFIC ESTERASE. (□) 1,25-$(OH)_2D_3$; (●) Δ^{22}-24,24,24-TRIHOMO-1,25-$(OH)_2D_3$;(○)24-HOMO-1,25-$(OH)_2D_3$;(■)24,24-DIHOMO-1,25-$(OH)_2D_3$;(△) Δ^{22}-24,24-DIHOMO-1,25-$(OH)_2D_3$.

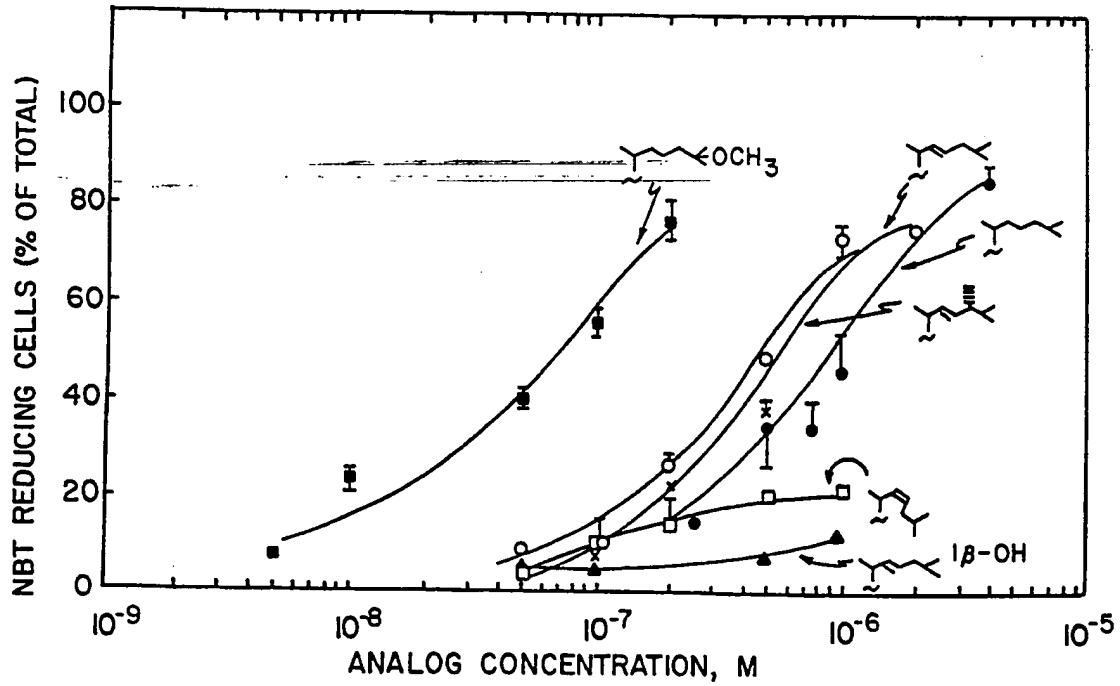
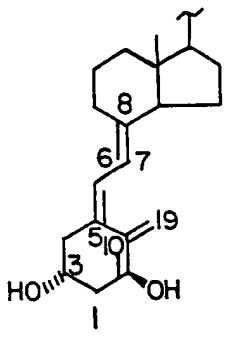


FIG. 7 ●, 1α-OH-D_3; ○, Δ^{22}-TRANS-1α-OH-D_3; x, 1α OH-D_2; ■, 1α OH-25-OCH_3D_3; ▲, 1β-OH-D_3; □, Δ^{22}-CIS-1α-OH-D_3



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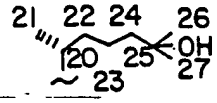
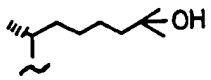
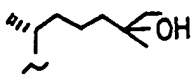
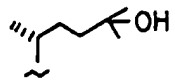
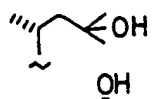
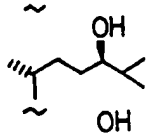
Ia 

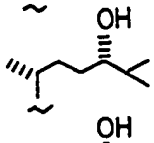
Ib 

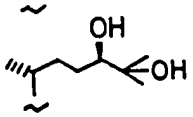
Ic 

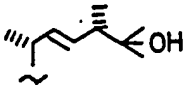
Id 

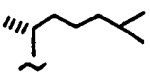
Ie 

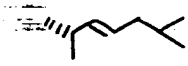
If 

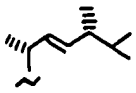
Ig 

Ih 

Ii 

Ij 

Ik 

Il 

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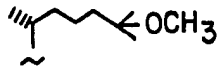
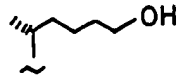
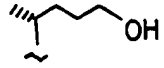
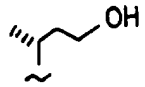
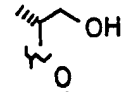
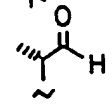
In 

Io 

Ip 

Iq 

Ir 

Is 

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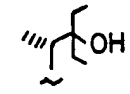
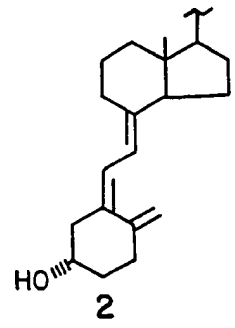
Iv 



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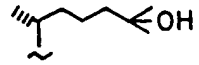
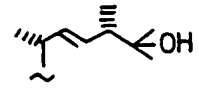
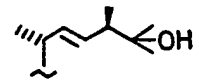
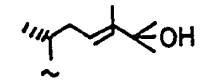
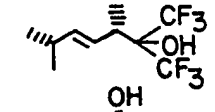
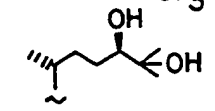
2a 

2b 

2c 

2d 

2e 

2f 

I: Iα-HYDROXYLATED ANALOGS. 2: 25-HYDROXYLATED ANALOGS. UNLESS OTHERWISE NOTED, STEREOCHEMISTRY OF THE NATURAL HORMONE IS: 3β -OH, Iα-OH, AND 5,6-CIS.

FIG. 6

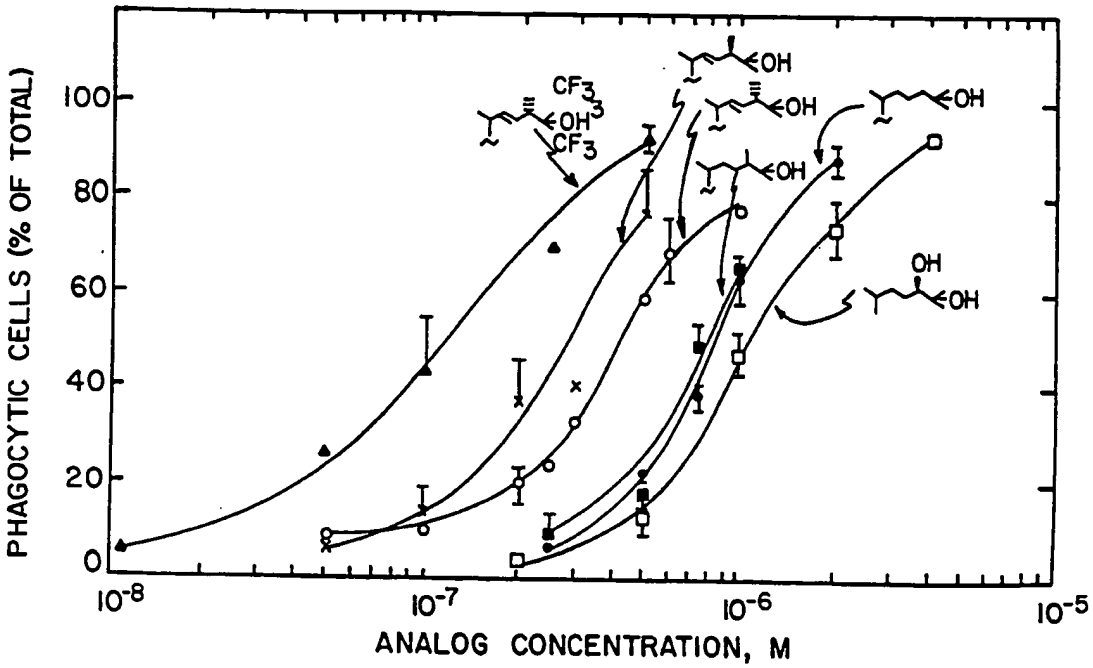


FIG. 8 •, 25-OH-D_3; ○, 25-OH-D_2; x, EPI-25-OH-D_2; ■, Δ^{23}-25-OH-D_2; ▲, 26,27-F_6-25-OH-D_2.

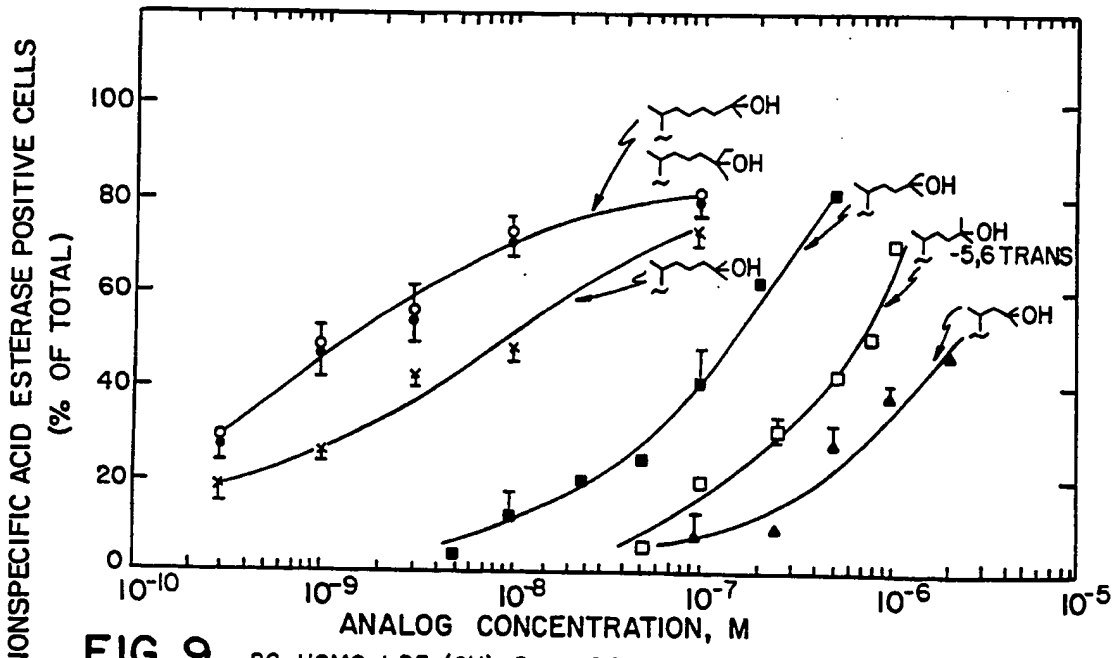


FIG. 9 •, 26-HOMO-1,25-$(OH)_2D_3$; , 24-HOMO-1,25-$(OH)_2D_3$; x, 1,25-$(OH)_2D_3$; ■, 24-NOR-1,25-$(OH)_2D_3$,5,6-CIS; ▲, 23,24-NOR-1,25-$(OH)_2D_3$; □, 24-NOR-1,25-$(OH)_2D_3$,5,6-TRANS.

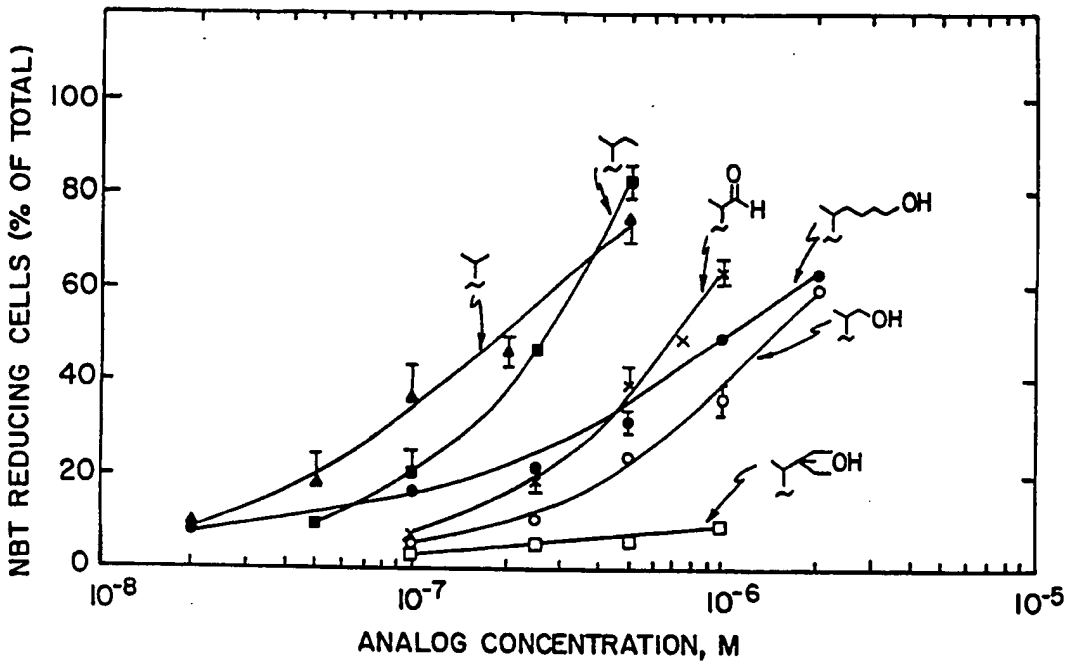


FIG. 10 •, 26,27-BISNOR-1,25-$(OH)_2D_3$; o,23,24,25,26,27-PENTANOR-1,22-$(OH)_2D_3$; x,24-al-1α-HYDROXYPREGNACHOLECALCIFEROL; ■, 1α-OH-BISHOMOPREGNACHOLECALCIFEROL; ▲, 1α-OH-HOMOPREGNACHOLECALCIFEROL.

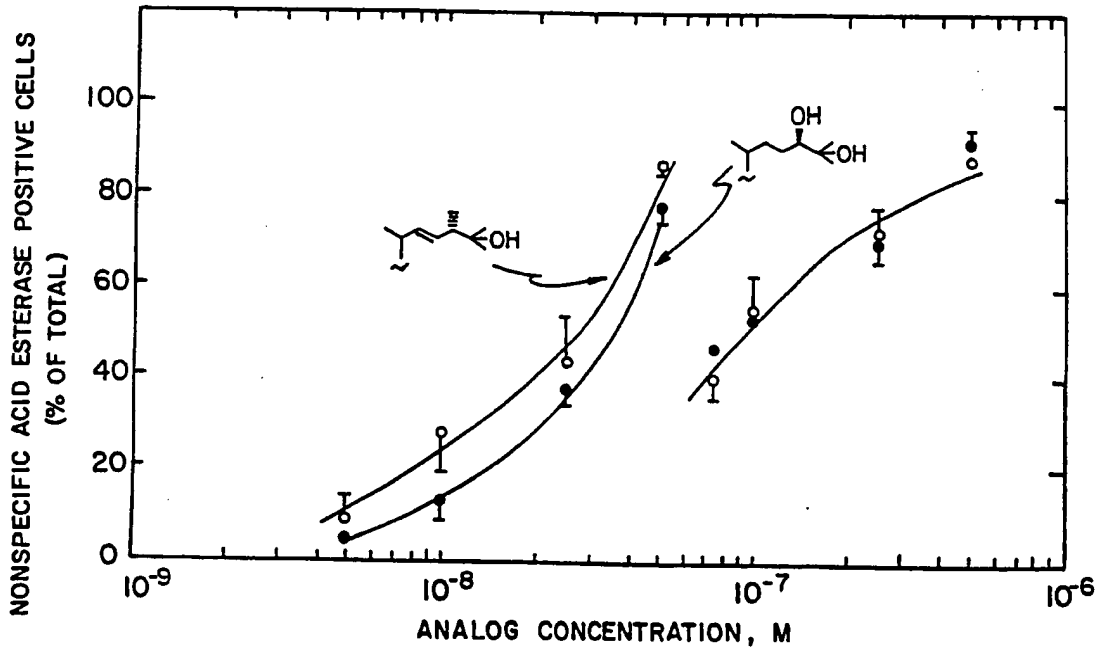


FIG. 11

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/05134

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: A 61 K 31/59

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	JNCI, vol. 78, no. 6, June 1987, Y. Nakao et al.: "Effect of 1alpha, 25-dihydroxyvitamin D_3 on proliferation of activated T-cells and established human lymphotropic virus type I-positive T-cell lines", pages 1079-1089, see the whole article	40-78
A	Science, vol. 224, no. 4656, 29 June 1984, C.D. Tsoukas et al.: "1,25-dihydroxyvitamin D_3: A novel immunoregulatory hormone", pages 1438-1440, see the whole article	40-78
A	Clinical Nephrology, vol. 16, no. 3, 1981, H. Miyakoshi et al.: "Immunological effects of 1alpha-hydroxy-	40-78

./.

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20th December 1990	30.01.91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	J. Toribio — Juria TORIBIO

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
	cholecalciferol (1alpha-OH-D_3) and its metabolites", pages 119-125, see the whole article	
	--	
X	WO, A, 86/02527 (THE GENERAL HOSPITAL CORPORATION) 9 May 1986 see claims	40-78
	--	
A	US, A, 4749710 (TRUITT et al.) 7 June 1988 see column 1, line 10 - column 2, line 38; table 4; examples 26-31; claims	40-78
	--	
A	US, A, 4225596 (DeLUCA) 30 September 1980 see the whole document	40-78
	--	
A	GB, A, 2126234 (WISCONSIN ALUMNI RESEARCH FOUNDATION) 21 March 1984 see the whole document	40-78
	--	
A	WO, A, 86/06255 (WISCONSIN ALUMNI RESEARCH FOUNDATION) 6 November 1986 see page 2, line 18 - page 3, line 34; claims	40-78

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers ...*..., because they relate to subject matter not required to be searched by this Authority, namely:

* - claims 1-39,79-95
see PCT - Rule 39.1(IV): methods for treatman of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers ....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9005134 SA 40528

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 18/01/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A- 8602527	09-05-86	US-A-	4728643	01-03-88
		EP-A-	0202276	26-11-86
		JP-T-	62501073	30-04-87
US-A- 4749710	07-06-88	None		
US-A- 4225596	30-09-80	None		
GB-A- 2126234	21-03-84	US-A-	4428946	31-01-84
		AU-B-	571846	28-04-88
		AU-A-	1826383	23-02-84
		BE-A-	897377	14-11-83
		BE-A-	897378	14-11-83
		CA-A-	1225593	18-08-87
		DE-T-	3390126	10-01-85
		FR-A,B	2530470	27-01-84
		FR-A,B	2530624	27-01-84
		GB-A,B	2124080	15-02-84
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		WO-A-	8400549	16-02-84
		AU-A-	1821383	23-02-84
		CH-A-	661047	30-06-87
		DE-T-	3390125	20-09-84
		NL-T-	8320218	01-06-84
		WO-A-	8400550	16-02-84
WO-A- 8606255	06-11-86	AU-A-	4537389	08-03-90
		AU-B-	599912	02-08-90
		AU-A-	5772786	18-11-86
		EP-A-	0218714	22-04-87
		GB-A,B	2185017	08-07-87
		GB-A,B	2212803	02-08-89
		JP-T-	62502545	01-10-87
		US-A-	4940700	10-07-90
		US-A-	4800198	24-01-89

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